Effects of Hypoxia on Detoxicating Enzyme Activity and Expression in HT29 Colon Adenocarcinoma Cells

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Abstract

Resistance of hypoxic tumor cells to ionizing radiation and cytotoxic drugs has been attributed to changes in the reactivity and/or the half-times of reactive species in the altered redox environment. Exposure of eukaryotic cells to such hypoxic conditions results in the induction of the synthesis of several unrelated proteins. To investigate further the phenomenon of hypoxic cell resistance to cytotoxic drugs, we examined the effects of hypoxia on the expression of a group of enzymes involved in drug metabolism. Exposure of HT29 colon carcinoma cells to hypoxia resulted in a marked increase in the activity of DT-diaphorase and in glutathione content. The activity of glutathione transferase was not increased by this treatment. The response was proportional to the duration of hypoxia. After the cells were exposed to hypoxic conditions for 8 h, followed by restoration of an oxic environment, the elevation in enzyme activity and glutathione content reached a peak at 48 h (40 h after the restoration of an oxic environment) and returned to baseline at 72 h. Elevation of steady-state levels of DT-diaphorase and γ-glutamylcysteine synthetase mRNA followed a similar time course, with >10-fold increases over oxic cells at 24 h. The elevation of DT-diaphorase mRNA content was found to result both from transcriptional induction and from increased message stability. The magnitude and persistence of elevated detoxicating enzyme activity following a relatively short hypoxic exposure followed by reoxygenation suggest a novel potential mechanism of resistance to cytotoxic drugs in hypoxic tumors.

Introduction

The common slow-growing tumors are resistant to most cytotoxic drugs (1). Recent work has characterized the cellular basis of this resistance. Alterations in pharmacological determinants of drug action, including transport across the plasma membrane, cytoplasmic cellular defense mechanisms, and repair of DNA lesions, have been associated with the acquired resistance phenotype (2). In vivo, additional impediments to drug action include poor perfusion of parts of the tumor, remoteness of cells from capillaries because of disordered angiogenesis, and elevated intratumoral hydrostatic pressure (3). These factors result in varying degrees of hypoxia. In solid tumors, substantial hypoxic fractions limit the effectiveness of radiation and chemotherapy (4).

Exposure of eukaryotic cells to a hypoxic environment results in adaptive changes, including altered expression of various proteins (5). The earliest identified were the "oxygen-regulated proteins" identified by Sutherland (6) as being overexpressed in hypoxic cells. The overexpression of certain of these proteins is common to the cellular response to stresses of other types (7). For example, peptide analysis has established that two of them (ORP 80 and/or ORP 100) are identified with proteins of about the same molecular weight that are induced by glucose deprivation (8). Additional effects of more prolonged periods of hypoxia have been identified by Stoler et al. (9). In rat fibroblasts, cessation of DNA synthesis is followed by adaptive changes in glucose metabolism and transcriptional activation of VL30 retrotransposon elements (9).

Many of the changes resulting from hypoxic exposure have features in common with the UV response which results in the induction of expression of several ubiquitous transcription factors, for which binding elements exist in the promoter region of various genes (10, 11). We have investigated the effects of hypoxia on the activities of several detoxicating enzymes, altered activities of which have been associated with drug resistance. In cultured human colon carcinoma cells, elevated activity of DT-diaphorase and increased glutathione content follow hypoxic exposure and continued culture under normally oxic conditions. We demonstrate that the elevated enzyme activity results from both transcriptional induction of the genes encoding these enzymes and stabilization of their mRNA.

Materials and Methods

Cells and Reagents

The HT29 colon adenocarcinoma cell line was grown in Dulbecco's modified essential medium, with 10% fetal bovine serum added. The cells were grown in 500-ml glass milk dilution bottles (Corning, New York) at 37°C in 5% CO2/95% air. Cells were passaged weekly and have a doubling time of 20 h. Reagents were purchased from Sigma, unless otherwise stated.

Hypoxic Exposure

Single-cell suspensions were plated at moderately high density (1.0-2.0 × 106 cells/bottle) and allowed to reach 30-40% confluence. Flasks for hypoxic exposure were sealed with rubber stoppers and insufflated through needles with O2-poor (<1 part in 10 billion) N2 for 2 h. Following removal of the needles, the flasks remained sealed for an additional 6 h. The hypoxic exposure was terminated by removing the rubber stoppers, which were then replaced with conventional caps, and cells grown further under normally oxic conditions were harvested at various times thereafter. Oxic cells grown under identical conditions were harvested at each time point, since prolonged cell culture results in altered expression of several of the enzymes (3). Cell viability following an 8-h treatment was measured by nitroblue tetrazolium exclusion and measured: oxic cells, 93.4 ± 0.2% (mean ± SE); hypoxic cells, 86 ± 1.5%.

Enzyme Activities

For enzyme assays, cells were washed twice in ice-cold PBS and harvested using a rubber policeman. The cell pellet was stored dry at -70°C until analysis. Upon thawing, cells were resuspended in PBS and lysed by sonication. The lysate was centrifuged at 12,000 × g for 15 min, and the supernatant was used in subsequent assays.

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The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. 1Supported by Grants CA 06972 and CA 49820 from the National Cancer Institute and 2To whom requests for reprints should be addressed, at Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111. 3P. J. O'Dwyer, manuscript in preparation. 4The abbreviations used are: PBS, phosphate-buffered saline; GSH, glutathione; ATA, aurintricarboxylic acid; SSC, standard sodium citrate; SDS, sodium dodecylsulfate; GCS, γ-glutamylcysteine synthetase; DTD, DT-diaphorase.
DT-diaphorase. DT-diaphorase was measured by a spectrophotometric assay in which the rate of reduction of 2,6-dichlorophenol indophenol was monitored at 600 nm (12). DT-diaphorase activity was calculated as the proportion of the total rate that is inhibited by 20 μM dicumarol. Results were normalized to protein by the Bradford assay (Bio-Rad, Richmond, VA).

Glutathione. Glutathione was measured by a modification of the method of Griffith (13), in which the rate of formation of a GSH conjugate of 5,5′-dithio-bis(2-nitrobenzoic acid) was determined spectrophotometrically. GSH concentrations were determined by reference to a standard curve (GSH, 0.05–5 nmol/ml) which was run with each batch of samples. Samples were precipitated by the addition of 12% 5-sulfosalicylic acid in a 1:3 ratio. The reaction was conducted in 0.2 mM NADPH-0.6 mM 5,5′-dithio-bis(2-nitrobenzoic acid), at pH 7.5. Absorbance was monitored at 412 nm for 3 min at 25°C, and results were derived from the standard curve and expressed per mg of protein.

Glutathione Transferase. Glutathione transferase was measured as described by Habig and Jakoby (14). The formation of a glutathione conjugate of 1-chloro-2,4-dinitrobenzene was monitored at 340 nm for 3 min at 25°C in a Beckman DU7 spectrophotometer with a Kinetics 3 package. The reaction was conducted in 1 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene. Results are expressed as specific activity in nmol/min/mg protein.

RNA Preparation and Northern Analysis

Total cellular RNA was extracted from cultured cell lines using a modified single step acid guanidium isothiocyanatephenol-chloroform extraction method (15). Briefly, the cells were washed once in PBS, and lysed in a solution containing 4 mM guanidium isothiocyanate-50 mM Tris-HCl, pH 7.6-10 mM EDTA-2% sarkosyl-0.1 M β-mercaptoethanol. The lysate was shaken vigorously in turn with 350 μl of 0.2 M sodium acetate-0.1 mM ATA, pH 4.0, followed by 3.5 ml of phenol saturated with 0.2 M sodium acetate-0.1 mM ATA, pH 4.0, followed by 700 μl of chloroform/isoamyl alcohol (24:1, v/v). This final suspension was chilled on ice and then centrifuged at 10,000 × g for 20 min at 4°C. RNA was precipitated from the aqueous layer with an equal volume of isopropanol and dissolved in RNA storage buffer (1.25 × 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer-62.5% deionized formamide). The RNA was reprecipitated with 1:10 volume of 3 M sodium acetate and 2 volumes of 100% ethanol, resuspended in RNA storage buffer, and stored at −20°C. Total RNA was denatured by adding 1.5 volume of 37% formaldehyde and heating at 60°C for 20 min. RNA (15–20 μg/lane) was separated in 1% agarose gel and transferred to nylon membranes (Magna NT: Micron Separations, Inc., Westboro, MA), using 10× SSC as the transfer buffer. When transfer was complete, the filter was dried at room temperature and baked for 1 h at 80°C in a vacuum oven.

Hybridization Conditions

Filters were prehybridized at 42°C for 2 h in a solution containing 50% formamide, 5× SSPE, 2× Denhardt’s reagent, and 0.1% SDS. 1× SSPE is 180 mM NaCl-10 mM sodium phosphate-1 mM Na2EDTA, pH 8; and 2× Denhardt’s reagent consists of 0.02% bovine serum albumin-0.02% polyvinylpyrrolidone-0.02% Ficoll. Probes were labeled by nick translation to give a specific activity of 1.5 x 108 cpm. The denatured radiolabeled probe was added directly to the prehybridization fluid. Hybridization was continued overnight at 42°C. The washing conditions were as follows: 20 min at room temperature in 1× SSC-0.1% SDS, 20 min at 55°C in 0.1× SSC-0.1% SDS, and 20 min at 55°C in 0.1× SSC-0.1% SDS. Autoradiography was carried out at −70°C for 3–7 days. The blot was subsequently stripped in boiling water and reprobed with β-actin. Densitometric scanning of the autoradiographs with normalization of values to the intensity of β-actin labeling provided a measure of relative expression of the mRNA of interest.

Nuclear Preparations

Nuclei were prepared from cells grown to 80% confluency essentially as described by Celano et al. (16). The cells were washed in ice-cold PBS and scraped into 5 ml of buffer A (20 mM Tris-HCl, pH 7.4-10 mM NaCl-3 mM MgCl2), after which they were made 0.1% by volume with Nonidet P-40. Cells were vortexed, and the plasma membrane was lysed in a sterile Dounce homogenizer on ice (10 strokes). The nuclei were pelleted at 1000 × g for 10 min at 4°C, washed in cold buffer A, and counted. The nuclear pellet was resuspended either in storage buffer (40% glyceral, 50 mM Tris-HCl, pH 8.3-5 mM MgCl2-0.1 mM EDTA) at 5 × 108 nuclei/ml or immersed in liquid nitrogen and stored at −70°C. For immediate assay, the nuclei were resuspended in transcription buffer (35% glyceral-10 mM Tris-HCl, pH 7.5-5 mM MgCl2-80 mM KCl-0.1 mM EDTA).

Run-on Assay

A simplification of the run-on assay described by Greenberg (17) was used (18). We used 106 nuclei/reaction, in a total volume of 200 μl in transcription buffer with 4 μM ATP, GTP, and CTP, and 200 μCi of [α-32P]UTP (Amersham, Arlington Heights, IL; 3000 Ci/nmol) at 26°C for 10 min. Nuclei were digested with 10 μl of ribonuclease-free DNase 1 (source; 10 mg/ml, made ribonuclease free using the iodacetate method) and 10 μl of calcium chloride (20 mM) at 26°C for 5 min. Samples were then treated with 2 μl of proteinase K (10 mg/ml, 15 μl of 10 × 5% SDS-50 mM EDTA-10 mM Tris-HCl, pH 7.4, and 5 μl of yeast RNA (10 mg/ml) at 37°C for 30 min. Nuclear RNA was isolated by the addition of 550 μl of 4 mM guanidium thiocyanate-25 mM sodium citrate, pH 7.0-0.5% Sarkosyl-0.1 M 2-mercaptoethanol-90 μl of 2 M sodium acetate, pH 4.0, containing 0.1 mM ATA, pH 4.0, followed by 900 μl of phenol equilibrated with ATA, and finally 180 μl of chloroform/isoamyl alcohol (24:1). This mixture was incubated on ice for 15 min and centrifuged for 15 min at 12,000 × g at 4°C, and the aqueous layer was transferred to a clean tube. To the aqueous layer was added an equal volume of isopropanol, and following incubation for 1 h at −70°C, the RNA was pelleted at 12,000 × g at 4°C. The pellet was again dissolved in 300 μl of the guanidium thiocyanate solution, and reprecipitated with 300 μl of isopropanol. After the sample was centrifuged, the pellet was washed in 70% ethanol and dissolved in sterile 10 mM Tris-HCl, pH 7.2-1 mM EDTA-0.1% SDS.

Immobilization of DNA Plasmids and Hybridization

Quantitation of the known activity of the genes of interest was measured using slot blots. The DNA probes were isolated from the plasmids using appropriate restriction enzymes. The following probes were used for these studies: (a) the 2-kilobase PstI actin insert in pBR322, (b) the 1.4-kilobase human DT-diaphorase insert in PUC 18, (c) the 3.7-kilobase NotI γ-glutamyl-cysteine synthetase insert in PBluescript (Stratagene). The probes were denatured by boiling and blotted (2 μg of DNA/blot) onto a pretwet nylon membrane (Magnat NT: Micron Separations, Inc., Westboro, MA), using 10× SSC as the transfer buffer. When transfer was complete, the filter was dried at room temperature and baked for 1 h at 80°C in a vacuum oven.

mRNA Stability

HT29 colon adenocarcinoma cells were seeded into 500-ml milk dilution bottles at 2 x 106 cells per bottle and allowed to grow to ~50–60% confluency. Cells were then treated with 8 h of hypoxia as previously described. At the end of the hypoxic exposure, cells were returned to oxic conditions for 16 h, when 100 μg/ml actinomycin D were added to hypoxic and oxic flasks. Cells receiving no actinomycin D were treated in parallel. RNA was extracted, separated on 3 ml of hybridization buffer and hybridized to the filters for 24 h at 42°C. Filters were washed in 2× SSC-1% SDS at 65°C for 1 h and 0.1× SSC-0.1% SDS at room temperature for 1 h. Autoradiography was performed at −70°C, and quantitation of the results was achieved by densitometric scanning and normalized to the signal for β-actin.

Results

Exposure of HT29 human colon adenocarcinoma cells to hypoxia for 8 h led to a pronounced increase in the activity of the detoxicating enzyme DT-diaphorase and in glutathione content (Figs. 1 and 2). Treated cells showed no increase in the activity of glutathione trans-
HYPOXIA AND DETOXICATING ENZYMES

content in the hypoxic cells. The time course of elevated mRNA content was parallel to that of DT-diaphorase activity, indicating a close correspondence between mRNA content and translation. The elevation of GSH content lagged somewhat behind γ-GCS mRNA levels, suggesting a complex response to redox stress, possibly involving accelerated turnover of GSH. Thus, the effects of hypoxia appeared to result primarily from an increase in steady-state mRNA content for the detoxicating enzymes.

The very striking changes in DT-diaphorase mRNA content led us to use this assay to investigate the dose-response characteristics of the hypoxic response. Cells were exposed to varying durations of hypoxia and assayed for mRNA content at the end of the period of hypoxia (without reoxygenation). Fig. 4 shows that the elevation of DT-diaphorase mRNA content was maximal at 8 h of hypoxia, sustained

ferase during the period tested. This is of interest because others have reported coregulation of DT-diaphorase and glutathione transferase following exposure of HepG2 cells to phenolic antioxidants (19).

The activities of these enzymes and glutathione content did not increase during the period of hypoxic exposure. In fact, slight decreases in activity and in glutathione content were detectable during and at the end of hypoxic exposure (Fig. 1). The elevation in DT-diaphorase activity was maximal at 24 h following exposure and remained elevated at 48 h. Thereafter, activities returned to those of oxic cells by 72 h (Fig. 2). The time course of glutathione elevation was similar. The observed time course is consistent with effects produced during hypoxia, during reoxygenation, or both.

To determine the mechanism of increased enzyme activity, we isolated total cellular RNA from HT29 cells at various times after hypoxic exposure. Northern blot analyses revealed elevated content of mRNA for DT-diaphorase (Fig. 3). The blots were also probed for γ-GCS, the rate-limiting enzyme in glutathione synthesis, the expression of which correlates well with measured glutathione content in various cell lines (20). Similarly, we found elevated γ-GCS mRNA

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Fig. 1. Change in glutathione content in human HT29 cells exposed to hypoxia for 8 h, followed by continued culture under oxic conditions, or cultured under oxic conditions throughout. Points, mean results from three separate experiments; bars, SE.

Fig. 2. Change in activity of DT-diaphorase in human HT29 cells exposed to hypoxia for 8 h, followed by continued culture under oxic conditions, or cultured under oxic conditions throughout. Points, mean results from three separate experiments; bars, SE.

Fig. 3. Northern blot analysis of the effects of hypoxia on steady-state levels of DTD and γ-GCS mRNA in human HT29 cells. A, total RNA from oxic (C), hypoxic (H) cells hybridized to a human DTD cDNA probe. The figure shows relative activity of mRNA of β-actin, an internal control for equal RNA loading. In B, autoradiogram shown in A was quantitated by laser densitometry, and the ratio of hypoxic/oxic was determined by the relative induction of DTD and γ-GCS to the level of the β-actin signal in hypoxic/oxic.
at 12 h, but less pronounced at 16 h, possibly reflecting toxicity from prolonged severe hypoxia.

The effects of hypoxia in increasing steady-state mRNA content for these detoxicating enzymes may have resulted from either activation of transcription or inhibition of degradation of mRNA or both. Evidence for transcriptional activation was sought using the nuclear run-on assay. A marked increase in the rate of transcription of RNA encoding DT-diaphorase was observed (Fig. 5). The time course of this effect differed markedly from the effect on steady-state levels. Induction of transcription was evident at the end of hypoxia, peaked at 24 h, and was similar to control at 48 h. The difference in time course suggests that exposure to hypoxia may also alter the stability of the DT-diaphorase message. When actinomycin D was used to inhibit new RNA synthesis, the rate of disappearance of the DT-diaphorase message differed in oxic and hypoxic cells (Fig. 6). The half-life of the message in oxic cells was approximately 1 h. In hypoxic cells a much slower decay in message levels was observed, although, as expected, the starting level was higher. We conclude that both transcriptional activation and stabilization of message contribute to the elevated steady-state DT-diaphorase mRNA levels.

Discussion

Hypoxia has long been recognized to confer resistance to ionizing radiation and more recently to cytotoxic drugs including alkylating agents and platinum compounds (1, 21). The basis of hypoxic cell resistance has been attributed to the altered intracellular redox status, which may change both the nature and half-times of radicals produced by ionizing radiation (22). In this study, we demonstrate that hypoxic cell resistance may have an additional contribution from the induction by hypoxia of some of the enzymes required for detoxication of xenobiotics. Overexpression of oxidoreductases may protect the cells from certain classes of DNA-damaging drugs (23). In addition, elevated GSH levels confer resistance to both radiation and cytotoxic drugs in several models (24, 25). For example, in cisplatin-resistant subclones derived from the A2780 human ovarian carcinoma, a 50-fold elevation in GSH was associated with a 1000-fold increase in resistance (20). Therefore, the alterations induced by hypoxia may have a substantial biological impact in terms of cellular protection.

The observed increase in enzyme activity appears to be a consequence of elevated mRNA content. The levels of steady-state mRNA encoding DT-diaphorase and γ-glutamylcysteine synthetase increase within 24 h of the hypoxic exposure and remain elevated for an additional 24—48 h. The contribution of altered enzyme turnover has not been addressed in this study but may be of some importance, since the enhancement of DT-diaphorase catalytic activity was substantially lower than the changes observed at the RNA level. In particular, the metabolic changes during hypoxic exposure (during which GSH levels decline by approximately 50%) may result in diminished protein stability and accelerated catabolism. Such changes may be involved in the signal transduction of the hypoxia response and would provide a plausible mechanism to mediate changes in gene transcription. The nuclear run-on assay (Fig. 5) shows that the elevation in steady-state mRNA content is accounted for in part by the induction of transcription. A significant posttranscriptional component is supported by the prolongation of the mRNA half-life following hypoxia.

Transcriptional induction of several genes has been described as a component of the hypoxic response. The oxygen-regulated proteins described by Sutherland (6) are induced upon hypoxic exposure. Recently, Price and Calderwood (26) demonstrated that the gadd (genes activated by DNA damage) are also induced by hypoxia. One of these, gadd 153 has an AP-1 site in its promoter region (27). Its induction following DNA damage is mediated through this site by
various conditions (5, 6, 19). The promoter regions of DT-diaphorase further upstream, several heat shock elements (28). We have previously demonstrated that hypoxia induces binding of the heat shock transcription factor to the elements in the promoter region of DT-diaphorase (29). It is not currently known whether the γ-glutamyl-cysteine synthetase promoter has such elements; however, these studies are currently in progress. Identifying which elements are shared may indicate which ones are critical to a specific response to hypoxia.

We have recently observed that a factor binding to the AP-1 site of DT-diaphorase occurs following exposure of HT29 cells to hypoxia. While the induction of DT-diaphorase expression occurs with some delay, nuclear preparations from hypoxic cells contain high levels of factors (specifically those of the jun family) that bind to the DT-diaphorase AP-1 site at earliest time points, preceding the detoxicating enzyme response. This temporal relationship suggests that elevated detoxicating enzyme activity is a consequence of the activation by hypoxia of immediate-early genes, which in turn act on response elements in the promoter regions of the genes encoding the detoxicating enzymes. Other known elements in the DT-diaphorase promoter are also candidates for mediating such responses and are being investigated for this function.

Such findings do not suggest that the response to hypoxia is specific. Several of the proteins are induced as part of the response to heat shock, UV radiation, ionizing radiation, and alkylating agents (5). The nature and extent of the protein induction varies with the nature of the stimulus. While the transduction of signals from these stimuli is undoubtedly specific to the nature of the stress, convergence of the pathways at some point before the induction of transcription factors may occur. The involvement of protein kinase C in the response to UV radiation provides a model: the expression of several stress proteins including gadd 153 following DNA damage is inhibited by H7, a protein kinase inhibitor (26). However, when a gadd 153-CAT construct is used as a reporter, CAT expression is not inhibited by H7. Thus, multiple pathways may determine the response, and as yet undiscovered negative regulators of stress responses may contribute to the eventual phenotype.

In conclusion, the finding that hypoxia results in the overexpression of detoxicating enzymes provides an additional dimension to hypoxic cell resistance. Since regions of tumors in vivo are variably hypoxic as a result of intermittent opening and closing of arterioles (30), this mechanism may contribute substantively to the constitutive resistance of tumors to drugs and radiation.

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