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Enhancement of Heme Oxygenase Expression and Activity in A431 Squamous Carcinoma Multicellular Tumor Spheroids[1]

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Abstract

We have investigated the effects of the growth of A431 human squamous carcinoma cells as three-dimensional aggregates (multicellular tumor spheroids) on the expression and enzyme activity of heme oxygenase (HO). We demonstrate that A431 squamous carcinoma cells grown as day 4 spheroids selectively increase the expression of heme oxygenase 1 (HO-1), caused, directly or indirectly, by three-dimensional cell-cell contact effects. Steady-state levels of both mRNA and protein are significantly enhanced in spheroids compared with day 4 monolayers (approximately 13-fold). Because of the similarity of apparent half-lives between monolayers (2.7 h) and spheroids (2.1 h), it appears that the increases are caused at least partly by altered transcriptional rates. Total HO enzyme activity, measured by carbon monoxide production, is also up-regulated (2.6-fold) in spheroids, compared to that in monolayers. This increase indicates that the up-regulation in HO-1 protein expression corresponds to an increase in functional enzyme levels. We propose that HO may play a more complex role in cellular metabolism than would be evident from studies using two-dimensional monolayer cultures.

Introduction

Multicellular spheroids are three-dimensional cellular or tissue aggregates grown *in vitro* on nonadhesive surfaces (agar) or in suspension. They can be considered to be intermediate between standard two-dimensional *in vitro* cultures and *in vivo* tissue. Spheroids have been studied extensively in developmental biology and in various aspects of tumor biology (1). In cancer research spheroids have been used to model micrometastases and intervascular regions of solid tumors (1, 2). Depending on the size of the spheroid, it is possible to study simple cell-cell interactions in small aggregates or the establishment of hypoxic, acidic, or hypoglycemic microenvironments in large spheroids (2). Studies using small spheroids have demonstrated that cell-cell contact increases resistance to stresses such as radiation (3), chemotherapeutic drugs (4), and hyperthermia (5).

Differences in genetic expression caused by cell-cell contact are not well characterized. Cellular interactions in dense monolayer cultures are known to up-regulate casein protein expression and secretion in mouse mammary epithelial cells (6) and to influence the expression of specific cytoskeletal and hepatic genes in rat hepatocytes (7). Our laboratory has recently shown that the transcription and synthesis of TGF-α[3] is enhanced in small, day 4 spheroids of A431 squamous carcinoma cells compared with that in monolayers (8). We postulated that cell-cell contact in combination with the three-dimensional architecture of the spheroids contributed to this observed increase attributed to the TGF-α/epidermal growth factor receptor autocrine loop. Others have observed that small spheroids (less than 100 μm) modulate the expression of genes in response to cytokines (9). In particular, cell-cell contact influenced both constitutive and induced expression of TGF-β mRNA in response to exogenously added TGF-β.

In separate studies designed to determine the effects of cell-cell contact on cellular responses to hypoxia, we observed a substantial up-regulation of an ORP in aerobic day 4 A431 spheroids compared with that in monolayer cultures. We have identified this protein as HO-1 (10). HO-1 and its isoform, HO-2, play essential roles in heme catabolism by cleaving heme to form biliverdin, iron, and CO. HO-1 is also thought to function as a stress protein because its expression is inducible in response to a wide variety of agents and environmental conditions including oxidative stress, heavy metals, and metabolic inhibitors (11). In this paper we discuss the possible physiological significance of the enhancement of HO-1 expression and total HO activity in three-dimensional tumor cell cultures.

Materials and Methods

Cell Culture and Spheroid Formation. A431 cells were maintained as previously described (8). Unless otherwise stated, monolayer cultures were prepared on day 1 by plating 3×10^5 cells into 100-mm tissue culture grade Petri dishes (Corning, Corning, NY) containing 10 ml of Dulbecco's modified Eagle's medium/10% fetal bovine serum and incubating them in 5% CO_2 at 37°C for 4 days. On day 4, monolayer cultures were approximately 80% confluent.

Day 4 spheroids were chosen for these studies because they have the maximum size distribution of spherical aggregates of A431 cells that can be obtained on a solid medium and because cell-cell contact is maximized without the establishment of microenvironments such as hypoxia (12). Because day 4 spheroid cultures contain fewer cells/dish than day 4 monolayer cultures, three dishes of spheroids were harvested compared with one dish of monolayers to ensure that similar numbers of spheroid and monolayer cells were lysed on day 4. To initiate spheroid growth suspensions of 1.2×10^6 cells were plated in 10 ml of Dulbecco's modified Eagle's medium/10% fetal bovine serum on 2% Noble agar (Difco Laboratories, Detroit, MI) prepared in 10% Dulbecco's modified Eagle's medium and coated on 100-mm Lab-Tek Petri dishes (Nunc, Inc., Naperville, IL) on day 1. Estimates of the sizes of randomly selected day 4 spheroids were calculated from the cubic means of orthogonal diameters measured using the eyepiece graticule of a phase-contrast inverted microscope. Diameters typically ranged from 80 to 120 μm.

Northern Blotting. For each RNA extraction, three plates of spheroids were pooled and centrifuged at $228 \times g$ for 3 min. Guanidinium isothiocyanate lysis solution (4 M, 3.5 ml) was added to the pellet. Total RNA was purified by cesium chloride ultracentrifugation. The medium was aspirated from the monolayer dishes, a total of 3.5 ml of lysis solution were added to all the dishes, and the samples were purified as described above.

DNA probes were labeled by the random primer technique using [α-^{32}P]-dCTP (Amersham, Arlington Heights, IL) and the Amersham Mega-Prime labeling system. Membranes were prehybridized for 2–12 h and probed (0.5–1.0 $\times 10^9$ cpm/μg) with a 1.1-kilobase *Eco*RI fragment derived from human HO-1 cDNA (13) (pHHO1; the gift of S. Shibahara, Tohoku University, Sendai, Japan). Membranes were also probed with an approximately 560-base pair

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[3] The abbreviations used are: TGF, transforming growth factor; HO, heme oxygenase; ORP, oxygen-regulated protein; cDNA, complementary DNA.

DNA fragment of a rat HO-2 cDNA amplified by polymerase chain reaction from a human brain cDNA library using primers based on published HO-2 cDNA sequences (14); (the gift of C. Bitler, SRI International). Levels of the β-actin mRNA internal standard were determined by using a 1.2-kilobase rat cDNA β-actin probe (15). Autoradiographs of the membranes were produced using Kodak XAR-5 film. Densitometry was accomplished by using a Lynx 4000 image analyzer (Applied Imaging, Santa Clara, CA).

Protein Labeling and Extraction. Spheroids were collected from three 100-mm plates and pooled into 35-mm agar-coated plates containing complete medium and 100 μCi/ml of [^{35}S]methionine (Trans ^{35}S-label, 1000 Ci/mmol; ICN Biochemicals, Irvine, CA). Each monolayer sample consisted of one 100-mm plate to which [^{35}S]methionine was added to a final concentration of 100 μCi/ml. Cells were incubated at 37°C in air containing 5% CO_2 for 12 h to achieve equilibrium labeling and then were placed on ice. The spheroid and monolayer samples were washed in cold phosphate-buffered saline and lysed in 0.4 ml lysis buffer as previously described (8).

Western Analysis for HO-1. Monolayers and spheroids were prepared and lysed as described above. Protein samples (15 μg/well) were resolved by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% discontinuous gels using a Hoeffer Scientific Instruments (San Francisco, CA) apparatus and electroblotted on Immobilon P membranes (Millipore Corp., Bedford, MA). HO-1 was detected with a polyclonal rabbit anti-rat heme oxygenase IgG antibody and visualized with a system composed of biotinylated goat anti-rabbit IgG (Vector Labs, Burlingame, CA), alkaline phosphatase/streptavidin conjugate (Vector Labs), and 5-bromo-4-chloro-3-indolyphosphate *p*-toluidine salt and nitroblue tetrazolium (GIBCO BRL, Gaithersburg, MD). The antibody was raised against a M_r 30,000 HO-1 protein expressed in *Escherichia coli* from a rat liver cDNA (gift of A. Wilkes, University of California, San Francisco, CA). The antiserum was prepared by the Berkeley Antibody Company (Berkeley, CA). Autoradiographs were obtained by placing the developed blots in X-ray cassettes with Kodak XAR-5 film and exposing overnight at room temperature.

HO Activity Measurements. Monolayer cells and spheroids were sedimented at 228 × *g* for 3 min. The cells and spheroids were resuspended twice in 3 ml of 0.1 M potassium phosphate (pH 7.4). The pellets were resuspended in 300 μl of the buffer and subjected to three brief freeze/thaw cycles (powdered dry ice/37°C water bath) to disrupt the cells. The lysate was immediately analyzed, in triplicate, for HO activity by measuring the rate of CO production, at 37°C, in the presence of 50 μM heme, 4.5 mM NADPH, and O_2. CO in the closed system was measured using a gas chromatograph with a reduction gas detector (Trace Analytical, Inc., Menlo Park, CA) (16). HO activity is expressed as nmol CO produced/h/mg of total cell protein as previously described (16).

Results and Discussion

The ratio of the mRNA level for HO-1 from spheroids to that from monolayers, determined by densitometry of autoradiographs of Northern blots, is designated Spd/Mono. The average value for day 4 A431 spheroids and day 4 monolayers is 13.8 ± 5.6 (SD) from five independent experiments. Similar Spd/Mono values (HO-1 mRNA) were obtained using day 4 spheroids and monolayers derived from the CaSki human squamous carcinoma cell line and from human keratinocyte primary cultures (data not shown). The size distribution within the spheroid population (80–120 μm, with a mean value of 100 μm) may account for the large SD. A typical autoradiograph of a Northern blot prepared for this study is shown in Fig. 1. This figure also includes mRNA profiles for day 4 monolayers and spheroids subjected to 12 h of hypoxia (≤300 ppm O_2) (10). The hypoxia experiments show that hypoxia induces HO-1 (ORP33) mRNA levels in human monolayer cultures as well as rodent (Chinese hamster ovary) cells (10) and that A431 spheroids respond to hypoxia by inducing HO-1 mRNA levels to a much greater extent than monolayers. For the experiment represented in Fig. 1, hypoxia resulted in approximately 3- and 15-fold increases in HO-1 mRNA levels for monolayers and spheroids, respectively, compared with aerobic controls. In contrast to the results for HO-1 mRNA, the aerobic A431 Spd/Mono ratios for HO-2 and β-actin mRNA levels were 0.85 ± 0.30 and 0.84 ± 0.36,

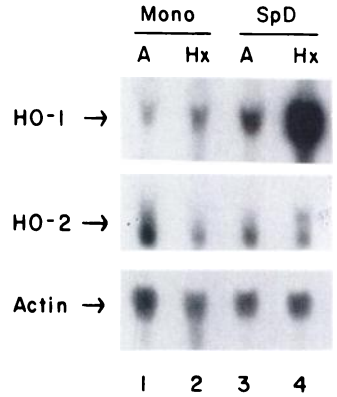

Fig. 1. Representative Northern blots of HO-1, HO-2, and β-actin mRNA. Total RNA (15 μg) was isolated and resolved as described in "Materials and Methods." *Lanes 1* and *2*, aerobic and hypoxic day 4 A431 monolayers, respectively; *Lanes 3* and *4*, aerobic and hypoxic day 4 A431 spheroids, respectively.

respectively, for the same five experiments (Fig. 1). Ongoing work in our laboratory indicates that the levels of seven other unrelated mRNA species are either slighly decreased or unchanged by growth of cells as three-dimensional aggregrates. These include triose phosphate isomerase, metallothionein II_a, c-*raf*-1, gaad 45, and cathepsin L,[4] ORP80 (also known as GRP78), and HSP70.[5] The results shown in Fig. 1 suggest that the regulation of transcription of HO-1 may involve different control mechanisms in response to cell-cell contact and hypoxic stress. Thus far we have observed that only HO-1 and TGF-α mRNA show increases in A431 spheroids compared with monolayers, suggesting altered regulation or physiological roles for the proteins in three-dimensional structures.

To determine the effect of monolayer cell density on the magnitude of Spd/Mono for HO-1, we performed experiments in which cells were plated at 3.3 × 10^4, 1 × 10^5, and 1.2 × 10^6 cells/100-mm dish and incubated for 4 days for comparison with day 4 spheroids plated at 1.2 × 10^6 cells. We found that different monolayer cell densities, ranging from sparse to confluent, had no significant effect on the relative levels of HO-1 mRNA. Only cells cultured as three-dimensional aggregates displayed increases in HO-1 message levels. This result is in contrast to the graded increases of TGF-α mRNA and protein observed in increasingly confluent A431 monolayers compared with spheroids (8).

It is highly unlikely that spheroids with diameters smaller than 150–200 μm could develop hypoxic microregions (12) which would contribute to a general induction of hypoxic stress proteins or ORPs, including HO-1. Indeed, we have observed that the synthesis rates of other major ORPs are unaffected by cell-cell contact in day 4 spheroids.[5] This same study showed that there were no significant inductions of ORP80 (identical to the glucose-regulated protein GRP78) mRNA or protein levels when A431 cells are grown as spheroids.

The apparent mRNA half-lives for HO-1 in exponentially growing day 4 A431 monolayer and spheroid cultures were measured by using actinomycin D (5 μg/ml of culture media). Total RNA samples were prepared at times from 0 to 5 h after addition of the RNA synthesis inhibitor, and the mRNA levels were determined by Northern filter hybridization. The apparent HO-1 message half-life is 2.73 ± 1.17 h (n = 4) for A431 monolayers and 2.09 ± 1.06 h (n = 4) for spheroids. The HO-2 and β-actin mRNA half-lives were also unaffected by the growth of these cells as multicellular spheroids. The similarities in the half-lives of HO-1 are consistent with the possibility that the induction of its mRNA steady-state levels in A431 spheroids is regulated transcriptionally rather than by message stabilization.

[4] Murphy *et al.*, unpublished results.
[5] Bourrat-Flock *et al.*, manuscript in preparation.

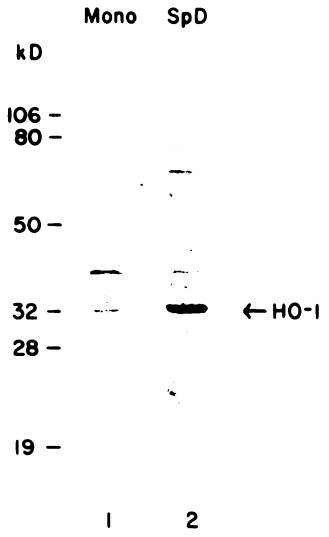

Fig. 2. Western blot analysis of HO-1 from A431 monolayers (*Lane 1*) and day 4 spheroids (*Lane 2*). Triton-soluble protein (20 μg) from each culture was separated on a one-dimensional sodium dodecyl sulfate-polyacrylamide gel and transferred to Immobilon membranes. The membrane was probed with a polyclonal rabbit antirat HO-1 IgG antibody and visualized as described in "Materials and Methods." The position of HO-1 is noted by an *arrow*.

Table 1 *Heme oxygenase activity measurements in cell lysates*

The numbers of individual experiments are given in parentheses. Spd/Mono = 2.60 ± 0.93.

	HO activity (nmol CO/h/mg protein)	
	Monolayers	Spheroids
$\bar{X}$ ± SD	0.53 ± 0.17 (5)	1.31 ± 0.42 (5)
Range	0.31–0.72 (5)	1.05–2.05 (5)

A one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis Western blot for HO-1 is presented in Fig. 2. The position of heme oxygenase is indicated by an arrow. This representative Western blot shows that the protein is maintained at approximately 13-fold higher levels when A431 cells are cultured as day 4 spheroids compared with exponentially growing monolayers. The difference is in contrast to the patterns of equilibrium protein synthesis rates, which are similar for the two cultures (approximately 1.25 × 10^3/h/μg protein). This is also valid when the incorporation rates are expressed per cell, because day 4 A431 monolayers and spheroids have approximately equal total protein per cell.[4] Diffusion of methionine is not a rate-limiting factor in small spheroids because the values of the protein synthesis rates are not different after dissociation with trypsin and subsequent metabolic labeling.

Total HO enzyme activity was measured to determine whether the enhancement of HO-1 protein expression in spheroids resulted in an increase in functional enzyme levels. Table 1 shows that the average value of Spd/Mono (activity ratio) is 2.60 ± 0.93 for five separate experiments. It was concluded that the measured CO was generated from the heme oxygenase reaction because addition of the HO inhibitor chromium mesoporphyrin (20 μM) (17) abolished NADPH-supported CO production. Positive controls for HO induction were obtained by adding heme (30 μM) to the cultures 16 h before lysis. For a typical experiment, heme induced HO activity levels in spheroids and monolayers by 1.5- and 3.6-fold, respectively. The difference between the expression of HO-1 mRNA and protein and that of the apparent enzyme activity levels is not surprising because the constitutive HO-2 also contributes to the total HO activity (18). Indeed, the oxidizing stress of UVA (320–380 nm) radiation has been shown to induce HO-1 mRNA expression by up to 13-fold, but the total enzyme activity increased by only 4- to 5-fold (19).

Heme oxygenase is a microsomal protein that is the rate-limiting enzyme in the oxidative catabolism of heme and produces biliverdin IX-α, $NADP^+$, Fe(III), and CO. Two distinct isozymes of HO have been identified (20), of which only HO-1 is inducible. A wide variety of stresses are known to induce the activity of HO-1, including X-ray and UV radiation, hormones, metal ions, organic metal complexes, disease states, therapeutic drugs, bacterial toxins (11), and hypoxia (10). Thus, it is likely that this enzyme plays a greater role than the maintenance of heme homeostasis, although the functional significance of HO induction under stress conditions remains to be elucidated.

It has been suggested that bilirubin, the product of the biliverdin reductase reaction, may act as a physiological antioxidant (21). In this view, cells grown as three-dimensional aggregates may require increased HO activity to confer additional antioxidant capacity. The enhancement of HO-1 expression and total HO enzymatic activity may facilitate the turnover of heme-containing proteins that are not needed in the cellular milieu of the spheroid. Another possibility is that CO, produced by HO catalysis of heme degradation, has a regulatory function in the tissue-like structures modeled by spheroids. CO has recently become the subject of intense investigation. This small molecule is postulated to behave as a messenger molecule much like nitric oxide, perhaps by activating guanylyl cyclase (22, 23) to produce increased amounts of the second messenger cGMP. CO has been demonstrated to inhibit platelet aggregation and causes relaxation of dog femoral, carotid, and coronary arterial preparations and rat coronary and aortic smooth muscle (see Ref. 23 for a review). More recently, evidence has been presented (22) that CO may function as a neurotransmitter. It is conceivable that CO levels in spheroids may positively regulate HO-1 expression, and consequently total HO activity, in neighboring cells. We are currently investigating the mechanism of HO-1 induction under both aerobic and hypoxic conditions. Culturing tumor cells, such as A431 squamous carcinoma cells, as small aggregates or multicellular tumor spheroids causes significant alterations of cellular HO-1 expresssion and total HO enzymatic activity levels compared with those observed in monolayer cultures. We suggest that HO may have a more complex function in cellular metabolism, stress responses, or cellular communication than would be evident from studies with two-dimensional cell cultures.

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