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RELATIONSHIPS BETWEEN THERMOTOLERANCE, OXIDATIVE STRESS RESPONSES AND INDUCTION OF STRESS PROTEINS IN HUMAN TUMOUR CELL LINES

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Abstract—Thermotolerance, resistance to oxidative stress and induction of stress proteins were examined in a panel of 10 human tumour cell lines. An inverse relationship was indicated between intrinsic thermotolerance (cell survival after treatment at 43.5° for 3 hr) and thermotolerance induced by pretreatment at 42.5° for 30 min. Similar levels of induction of hsp 70 were found in cell lines with high or low levels of intrinsic thermotolerance; induction of other stress proteins could not be detected. Cell survival following treatment with H_2O_2 correlated with that following streptonigrin treatment ($P < 0.05$). Pretreatment with buthionine sulphoximine or diamide synergistically increased the toxicity of heat, H_2O_2 and streptonigrin whereas reduced glutathione had the reverse effect. No direct correlation was found, however, between tolerance to heat and to oxidative stress, and hsp 70 was not induced by the latter. The stress protein heme oxygenase, detected by immunoblotting with the monoclonal antibody HO, was induced by H_2O_2 in melanoma cell lines but not in HeLa. Cadmium and arsenite ions, however, readily induced heme oxygenase in HeLa, indicating that in these cells induction of heme oxygenase by oxidative stress involves a different mechanism. Overall, the results suggest that tolerance to heat or oxidative stress in these cell lines may not necessarily be associated with the induction of heat shock proteins or heme oxygenase but that cell survival after both types of stress depends to a certain extent on cellular sulphydryls.

The exact mechanisms by which heat kills cells remains unknown, although cellular membranes, cytoskeletal structures, energy (respiratory) metabolism, protein synthesis or DNA may be a target for hyperthermic injury. A potential obstacle in fractionated heat treatments in the clinic is the phenomenon of thermotolerance, in which hyperthermic treatments can induce a transient resistance to subsequent heat exposures. This phenomenon has been observed in a wide range of organisms including human tumours, xenografts and cultured tumour cells [1–5]. Induced protection or thermotolerance has been suggested to be correlated with the appearance of heat shock proteins (hsps‡). Hsp 70 has been found to occur naturally in most cells and is induced in response to heat and other stresses, which suggests an important role in protection against stress and subsequent recovery from cellular injury [1–6]. For example, introduction of a human hsp 70 gene into rat fibroblasts led to increased synthesis of hsp 70 and a corresponding increase in intrinsic resistance to thermal stress [7]. Oxidative stresses such as H_2O_2, UV radiation, sodium arsenite and cadmium also induce the synthesis of hsp in human cells [8].

Polla *et al.* [9] demonstrated that generation of oxygen free radicals during erythrophagocytosis is associated with the synthesis of the classical hsp and a 32 kDa protein, since identified as heme oxygenase [10]. An increase in heme oxygenase will increase cellular capacity to generate both biliverdin, a singlet oxygen scavenger, and bilirubin, a potential antioxidant [11]. The agents which lead to the induction of heme oxygenase in cultured human fibroblasts [12] fall into two categories: oxidants and treatments that can generate active intermediates (UV-A, hydrogen peroxide and menadione); and agents which are known to interact with or modify cellular reduced glutathione (GSH) levels [butathionine-*S*,*R*-sulphoximine (BSO), arsenite, iodacetamide, diamide and cadmium]. GSH itself has been shown to play a role in thermotolerance and the induction of hsp [13–16]. In addition, the levels of lipid peroxide [17] and superoxide dismutase [18–20] increase during hyperthermia, and depletion of lipid peroxides by free radical scavengers prevents lipid peroxidation and induction of hsp by heat [21].

Burdon [17] reported that hyperthermia results in inhibition of protein synthesis in HeLa cells, an effect which was diminished in the presence of sodium azide or mannitol suggesting that the initial inhibitory effects may be due, amongst other things, to the damaging effects of oxygen-derived free radicals. These observations suggest that oxygen radicals and cellular antioxidant defences may play an important role in the pathogenesis of hyperthermic injury, and may share common mechanisms of induction and tolerance.

In this study a panel of human tumour cell lines

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‡ Abbreviations: BSO, buthionine-*S*,*R*-sulphoximine; GSH, reduced glutathione; GSSG, oxidized glutathione; hsp, heat shock protein.

was used to seek correlations between markers of heat and oxidative stress. Induction of hsp 70 and the oxidative stress protein heme oxygenase were also investigated.

MATERIALS AND METHODS

Cell culture. The origins of the cell lines are described in Table 1. Cultures were grown at 37° in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 5% foetal calf serum (v/v), penicillin (100 U/mL), streptomycin (100 μg/mL), and 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (3 mM), and maintained in a humidified atmosphere containing 5% CO_2/air. All cell lines were found to be free of mycoplasma by staining with Hoechst 33258.

Cell survival. For cell survival assay, cells were seeded into 96-well plates in duplicate at a density of 1×10^4 well and incubated overnight to allow attachment, followed by treatment. Cell survival was determined by thymidine incorporation in colonies 5–7 days after treatment. This method has been found to give results comparable to visual counting of colonies [22]. The cells were incubated for 4–6 hr in medium containing 5 μCi/mL [*methyl*-^{3}H]-thymidine (18.4 Ci/mol; Radiochemical Centre, Amersham, U.K.), washed in phosphate-buffered saline (50 mM phosphate, 0.1 M NaCl, pH 7.2), detached with 0.02% trypsin and washed onto glass fibre disks (Whatman GF/A) for liquid scintillation counting. Cell survival was calculated as a percentage of control cpm. Stock solutions of drugs were freshly prepared in culture medium for each experiment. Heat treatment was carried out by placing dishes in a humidified atmosphere containing 5% CO_2/air at the stated temperatures.

[^{35}S]*Methionine labelling.* Cells were grown to confluency in 10 mL petri dishes in normal foetal calf serum supplemented medium, washed with serum- and methionine-free medium and incubated with [^{35}S]methionine (30 μCi/mL) in the latter medium during heat treatment and subsequent recovery at 37°. Cells were harvested by scraping into ice-cold phosphate-buffered saline and pelleted. The pellet was sonicated in cell lysis buffer (10 mM dithiothreitol, 20% glycerol, 1% SDS, 10 mM Tris, pH 7.4 and 2 mM phenylmethylsulphonylfluoride) for 1 min. Lysates were then immersed in boiling water for 2 min. Aliquots were applied to 10% polyacrylamide gels and electrophoresed in 25 mM Tris containing 192 mM glycine and 0.1% SDS. Relative molecular mass standards were obtained from Pharmacia (Sydney, Australia). Gels were dried and autoradiographed for 2–3 days. Protein content was determined using bicinchoninic reagent (Pierce, IL, U.S.A.) and reading of the absorbance at 540 nm; bovine serum albumin was the protein standard.

Monoclonal antibodies. The monoclonal antibody HO to human heme oxygenase was derived using the peptide APGLRQRASNKVQDSAPCB chosen from the published sequence [23]. The peptide was coupled to diptheria toxin for immunization of BALB/c mice. The immunization and screening procedures have been described previously [24]. For immunoblotting, cell sonicates were subjected to SDS–PAGE and transferred to nitrocellulose. Immunoreactive bands were visualized by sequential incubations with HO-2 hybridoma supernatant, alkaline phosphatase-conjugated sheep antimouse antibody, and bromochloroindoyl phosphate/nitroblue tetrazolium substrate buffer, pH 9.6, as described [24].

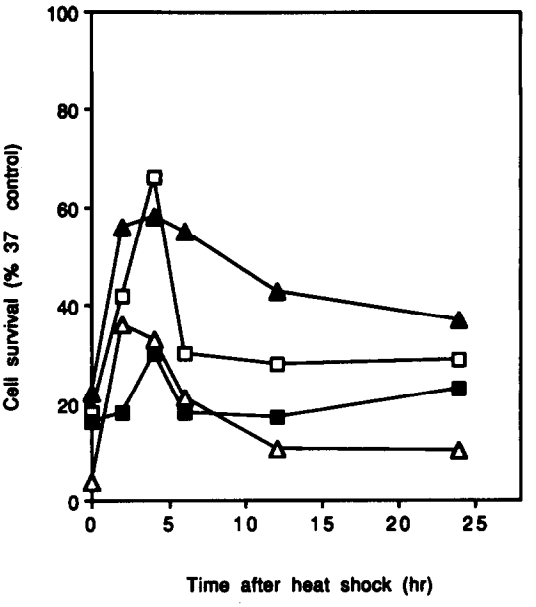

Fig. 1. Induction of tolerance to heat stress (43.5° for 3 hr) by exposure to heat shock (42.5° for 0.5 hr) at different times beforehand, cell survival being expressed as per cent of 37° controls. (□) HeLa; (△) MM96E; (■) MM418; (▲) MM253c1. Shock alone gave 80–100% survival in all cell lines. Points represent means of three experiments. SE <10% of each mean.

RESULTS

Kinetics of thermotolerance development in human tumour cells

The term heat shock refers to a brief, non-toxic treatment given with the aim of inducing cellular responses, whereas the term stress refers to a subsequent treatment of sufficient severity to show some toxicity when used alone. A heat shock of 42.5° for 30 min was sufficient to induce tolerance to a subsequent heat stress of 43.5° for 3 hr in four human cell lines. The heat shock itself was not toxic (100% survival). The heat stress alone resulted in 20–40% survival for HeLa, MM96E and MM418 cells compared with 2.5% survival for MM253c1 cells (Fig. 1 and Table 1). Figure 1 shows the thermotolerance ratio (ratio of the shock plus stress to the stress only survival) of cells with different intervals between heat treatments. The magnitude of the induced thermotolerance was dependent upon the recovery interval between the two heat treatments, thermotolerance reaching a maximum

Table 1. Survival of human tumour cells and fibroblasts exposed to heat, hydrogen peroxide or streptonigrin

Cell line	Origin	Ref.	% survival		
			Heat (43.5° for 3 hr)	H_2O_2 (300 μM for 30 min)	Streptonigrin (12 ng/mL for 1 hr)
JAM	Ovarian cancer	[25]	39	17	13
HeLa	Cervical cancer	[26]	29	5	8
MM96E	Melanoma	[26]	25	1.8	2.24
MM170	Melanoma	[27]	22	8.4	4.1
A2058	Melanoma	[28]	20	25	48
MM418	Melanoma	[26]	18.6	13	6.6
GG	Ovarian cancer	[25]	16	2	1.8
NFF	Neonatal foreskin fibroblasts	[26]	13	2.4	1.7
KJD-SV40	SV-40-transformed keratinocytes	[29]	9	42	42
CI80-13S	Ovarian cancer	[30]	7.7	14	4
MM253c1	Melanoma	[26]	4.5	20	34

Typical results from one of four experiments. SD replicates <10% of the mean.

Linear correlation coefficient for hydrogen peroxide vs streptonigrin toxicities, 0.856 ($P < 0.05$). For other potential correlations between treatments, $P > 0.05$.

in cells 2–4 hr after the heat shock and then steadily declining. This time-dependent phenomenon was seen in all four lines studied. Some variation in cell survival was found between experiments, presumably due to small differences in pH and in the level of hyperthermia achieved in the microtitre plate. All comparisons of cell lines and combination treatments were therefore carried out within the one experiment and treatments at a particular temperature were all conducted in the same plate.

Induction of stress proteins

To determine the role and kinetics of induction of hsps in the stress response in HeLa cells, the profile of newly synthesized proteins in heat-shocked cells (42.5° for 30 min) was analysed using [^{35}S]-methionine labelling. Cells were heat shocked and then incubated at 37° for various time intervals, before extraction. As Fig. 2 shows, a heat shock of 42.5° for 30 min induced synthesis of a protein at 70 kDa, assumed to be hsp 70. This treatment did not induce any of the other hsps observed previously in human cell lines [31]. Induction of hsp 70 was evident within 2 hr after treatment, increased up to 6 hr and was maintained at this level for 24 hr. The melanoma cell lines MM96E and MM253c1 produced similar increased in the level of hsp 70 (profiles not shown).

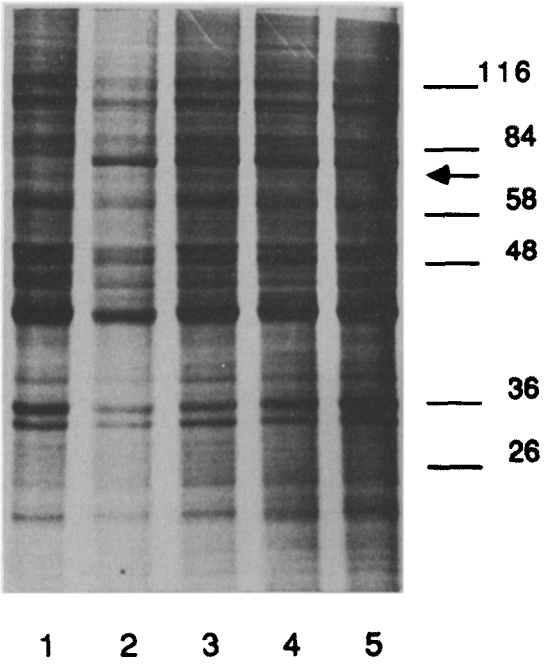

Fig. 2. Induction of hsp 70 in HeLa cells, detected by labelling cultures with [^{35}S]methionine at various times after heat shock (42.5° for 30 min) and separation of proteins by PAGE. Lane 1, 37° control; lane 2, 2 hr; lane 3, 6 hr; lane 4, 12 hr; lane 5, 24 hr.

Relationship between intrinsic thermotolerance and induced thermotolerance

The ability of 10 human tumour lines and a fibroblast strain to develop thermotolerance after heat shock was analysed using a 6 hr recovery period between the two heat treatments (Fig. 3). The response of normal fibroblasts (NFF) was in the mid-range of that exhibited by the tumour cells and there was no correlation between the tumour type and intrinsic or induced thermotolerance. Overall, the results indicated an inverse trend between the intrinsic and induced thermotolerance (linear correlation coefficient 0.7). Thus, cells most sensitive to heat stress acquired a higher degree of thermotolerance after heat shock.

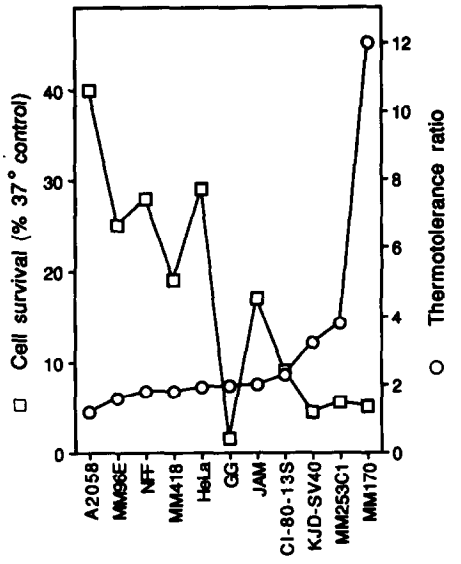

Fig. 3. Inverse relationship in different cell lines between intrinsic thermotolerance (□, cell survival after a stress of 43.5° for 3 hr) and thermotolerance induced by heat shock (○, 42.5° for 0.5 hr) given 6 hr before the stress treatment. Points represent means of duplicates. SD <10% of each mean. The thermotolerance ratio was calculated as % survival from shock plus stress/% survival from stress alone.

Cross-tolerance of cell lines to heat and oxygen radical-generating agents

The relationship between heat and oxidative stress was examined using hydrogen peroxide and streptonigrin as oxidative stress agents. Cell survival was determined after treatment with heat (43.5° for 3 hr), hydrogen peroxide (300 μM for 3 hr) or streptonigrin (12 ng/mL for 3 hr) (Table 1). There was a positive correlation between the sensitivities to hydrogen peroxide and streptonigrin, suggesting that killing by exposure of cells to hydrogen peroxide either added exogenously or generated intracellularly occurs via the same pathways. However, there was no correlation between the susceptibility of the tumour cells to heat and oxidative stresses.

To further study the relationship between oxidative and heat stress, the effect of heat shock on the ability of cells to resist oxidative stress was tested. Cells were stressed by either 300 μM hydrogen peroxide or 12 ng/mL streptonigrin with and without a prior heat shock treatment (42.5° for 30 min). The survival rates of cells exposed to heat shock prior to the hydrogen peroxide stress or streptonigrin stress were not higher than the stress alone, suggesting that heat shock does not protect from oxidative stress in the 11 lines tested (results not shown).

Role of sulphydryl agents in protection from heat and oxidative stress

HeLa exhibited higher survival rates than the melanoma lines MM96E and MM418 after treatment with BSO or diamide (Table 2) The effect of heat (43.5° for 3 hr) on cell survival following overnight pretreatment with these agents or with GSH is summarized in Table 2. GSH increased the heat resistance of HeLa from 17% to 64% survival. However, GSH did not protect the melanoma lines MM96E and MM418 from killing by heat. Addition of both GSH depletors to the culture medium prior to heat treatment resulted in a synergistic decrease in survival in all the lines studied. These results suggest that exogenous GSH does not necessarily protect against killing by heat, but that GSH deficiency sensitizes cells to heat.

Addition of GSH to the culture medium helped to protect all three cell lines from hydrogen peroxide toxicity (Table 2). BSO decreased the survival of H_2O_2-treated HeLa cells but did not affect the survival of the MM96E and MM418 cell lines. GSH was found to be protective against streptonigrin toxicity whereas BSO decreased the survival rates of HeLa and MM418. HeLa but not melanoma cells were sensitized to streptonigrin by diamide.

Induction of heme oxygenase

A monoclonal antibody was derived using a peptide sequence of human heme oxygenase as the immunogen. After primary screening on HeLa cells fixed in microtitre plates, one clone was found which yielded a 32 kDa band on western blotting. This corresponded to the relative molecular mass of heme oxygenase [10]. Further evidence for the identity of heme oxygenase as the antigen was obtained from cadmium chloride treatment of the cells in culture (50 μM for 30 min), where an increase occurred in HeLa cells over the following 6 hr, then slowly declined during the next 18 hr (Fig. 4). Sodium arsenite (50 μM for 30 min) also caused a similar time-dependent induction of heme oxygenase, although the level was not as high as that induced by cadmium chloride. Metabolic labelling with [^{35}S]-methionine was not sufficiently sensitive to detect an increase in heme oxygenase.

The effect of exogenous hydrogen peroxide upon the level of heme oxygenase was studied by incubating cells with 20 μM hydrogen peroxide for 30 min followed by 6 hr of recovery. This treatment increased the synthesis of heme oxygenase in the melanoma lines MM96E and MM253c1 but not in HeLa cells (Fig. 4). Heme oxygenase was not inducible in HeLa cells by hydrogen peroxide (10–1000 μM) at intervals of up to 24 hr after a 30 min treatment, or by streptonigrin (12 ng/mL for 30 min). Neither BSO nor diamide (each 100 μM for 18 hr) resulted in an increased expression of heme oxygenase (results not shown). HeLa, MM96E and MM253c1 cells treated at 42.5° for 30 min and left to recover for intervals of up to 24 hr showed no induction of heme oxygenase.

DISCUSSION

This report demonstrates that human non-melanoma and melanoma cell lines, like other cells studied so far [3–5], are capable of developing tolerance to otherwise lethal heat treatments by a non-lethal heat shock 6–24 hr beforehand. An inverse trend was found between the natural (intrinsic) resistance to heat and ability to acquire an induced tolerance to heat, in contrast to a study of five cell lines by Rofstad [4]. Reduction in pH during and between heat periods decreases the level

Table 2. Effect of GSH and GSH-depleting reducing agents upon subsequent response of human tumour lines to heat, hydrogen peroxide and streptonigrin stress

	Cell survival (% control)						
Cell lines	Stress	GSH*	GSH + stress	BSO†	BSO + stress	Diamide†	Diamide + stress
Heat (43.5° for 3 hr)							
HeLa	17‡	85	64 (15)	83	9 (14)	27	1.5 (5)
MM96E	37	76	11 (28)	35	3 (13)	15	0.03 (6)
MM418	48	65	26 (31)	69	5 (33)	18	4.1 (9)
H_2O_2 (110 μM for 3 hr)							
HeLa	16	82	34 (13)	86	2 (14)	76	7.6 (12)
MM96E	36	36	91 (13)	36	15 (13)	16	11 (6)
MM418	76	73	41 (56)	47	47 (36)	14	7 (11)
Streptonigrin (5 ng/mL for 1 hr)							
HeLa	56	82	70 (46)	86	47 (48)	82	42 (46)
MM96E	35	63	30 (22)	36	25 (9)	16	16 (6)
MM418	49	73	63 (36)	47	7 (23)	15	15 (7)

* 100 μM for 18 hr. Cells were then washed and immediately subjected to the stress treatments.
† 100 μM for 18 hr.
‡ Mean of duplicates. SD < 10% of each mean. Numbers in parentheses indicate % survival expected if the two treatments had an additive effect (product of the survivals from separate treatments).

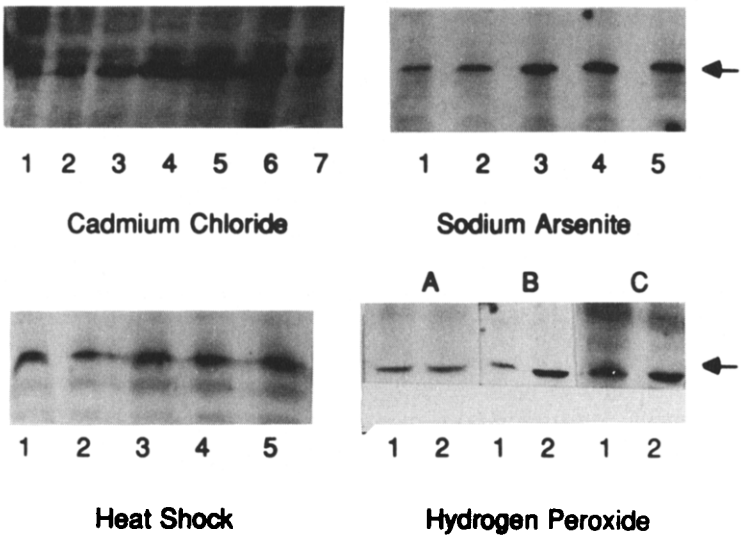

Fig. 4. Immunoblot analysis of the induction of heme oxygenase. Cadmium chloride (50 μM for 30 min) and HeLa: lane 1, 37° control; lanes 2–7, 0, 2, 4, 6, 12 and 24 hr after treatment, respectively. Sodium arsenite (50 μM for 30 min) and HeLa: lanes 1–5, 0, 2, 6, 12 and 24 hr after treatment, respectively. Heat shock (42.5° for 30 min) and HeLa: lanes 1–5, 0, 2, 6, 12 and 24 hr after treatment, respectively. Hydrogen peroxide (20 μM for 30 min) and HeLa (A), MM96E (B) and MM253c1 (C): control, lane 1; cells 6 hr after treatment, lane 2. The arrow indicates the heme oxygenase band (32 kDa).

of induced thermotolerance, which also decays more quickly in rapidly cycling cells [2]. Culture conditions such as pH may vary between experiments but cannot account for the consistently high degree of thermotolerance induced in the MM253c1 cells because all cell lines were seeded in the same microtitre plate for each experiment. The doubling time of this cell line was not significantly different from those of most of the other lines used, although it should be noted that the lack of inducible thermotolerance in HeLa cells was associated with failure to accumulate in G1 during hyperthermia rather than the length of the cell cycle [32]. It is therefore possible that protective genes are constitutively expressed in thermotolerant cells but require induction in thermosensitive cells such as MM253c1.

Involvement of hsps in the development of

thermotolerance is suggested by a number of experimental approaches including mutational [6] and kinetic [33–35] analysis of hsps. Other studies, however, showed that hsp synthesis by cells is neither a necessary nor sufficient condition for thermotolerance development, although there is evidence that the expression of hsp70 and thermotolerance is regulated by interrelated mechanisms [3, 36, 37]. Hsp 70, which is constitutively expressed in human cells, was elevated by heat shock to similar extents in all the cell lines studied. In contrast to previous experiments with HeLa cells [38], no induction of hsp 28 was observed in the present study. Synthesis of hsp 70 was increased as early as 2 hr and was maintained for up to 24 hr following the treatment. On the other hand, thermotolerance was maximal at 6 hr and slowly declined over the next 18 hr period. However, these results by themselves do not eliminate the possibility that hsp 70 synthesis may be involved in thermotolerance and cell recovery from thermal stress.

Hsps may play a role in the response to injurious agents other than heat. Many hsps are induced by other forms of stress such as anoxia, heavy metals and glucose deprivation and there is increasing evidence for a role as molecular chaperones [39]. There was no correlation in the present study between the heat and oxidative stress responses, and there was no cross-induction of tolerance. However, treatment with either BSO or diamide caused an increased thermosensitivity as well as susceptibility to killing by hydrogen peroxide, suggesting that free radicals are involved in heat-induced cell killing.

In studies of proteins induced by oxidative stress we found that heme oxygenase could be induced in HeLa cells by cadmium ions but not by H_2O_2 or other oxidative stress. A further example of the heterogeneous responses of cells to stress, this indicates that HeLa lacks part of the heme oxygenase induction mechanism yet does not suffer increased toxicity. Melanoma cells, which increase heme oxygenase synthesis in response to both types of inducers, failed to show increased levels of heme oxygenase after heat treatment. This is in agreement with previous evidence that heme oxygenase is not an hsp in human cells [40, 41]. On the other hand, it is noteworthy that Mitani *et al.* [42] showed that heme oxygenase mRNA is induced in certain human hepatoma cells but not in others by heat shock treatment. Furthermore, heat shock is a potent inducer of heme oxygenase mRNA in rodent cells [43–45].

The results from this study demonstrate that induction of hsp 70 and heme oxygenase is controlled through different mechanisms and that heme oxygenase in particular is not necessary for induced thermotolerance in human tumour lines. The only link found between heat and oxidative stress was through modulation of cell thiols. These observations support previous evidence [46] that thermotolerance is accompanied by an increase in GSH levels and conversely, depletion of GSH by diethylmaleate or inhibition by BSO affects the development of thermotolerance. These results implicate the GSH–GSSH cycle in protecting cells against heat and oxidative agents. Interestingly, Lengfelder and Fink [47] demonstrated that during hyperthermia the production of activated oxygen species is increased, thus suggesting that free radicals may be involved in heat-induced cell killing. Proteins with active SH groups that depend on GSH for maintenance of the reduced form might therefore be common targets for heat and oxidative stress. The question of whether the synthesis of such molecules is induced as an essential part of the stress response in cells such as MM253c1 remains to be determined.

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