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Antioxidant Enzymes and Survival of Normal and Simian Virus 40-transformed Mouse Embryo Cells after Hyperthermia

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ABSTRACT

Relative cell survival and activity of the free radical scavenging enzymes superoxide dismutase, catalase, and glutathione peroxidase were measured in cloned normal (MEA) and SV40-transformed (SVMEA) mouse embryo cells exposed at 44°C for 0–3 h. At 37°C, all three enzymes were 2–5 times higher in MEA than in SVMEA. Hyperthermia did not significantly alter enzyme levels in either cell line but selectively reduced transformed cell survival to less than 5% while relative survival of normal cells remained above 75%. The latter, however, could be reduced to 25% when normal cells were pretreated with 3 mM diethyldithiocarbamate, an inhibitor of copper- and zinc-containing superoxide dismutase. Similar treatment rendered SVMEA extremely thermosensitive. On the other hand, sublethal heat treatment (15 min at 45°C) of cultured cells resulted in a relative thermal resistance upon subsequent exposure to 45°C for 1–4 h. This induced thermotolerance was associated with a rise in antioxidant enzyme levels and both became significant only 4–6 h after the initial heat treatment. Induced enzyme and thermotolerance levels in transformed cells remained, nonetheless, far below those of normal cells. The data show that inherent (in MEA) as well as induced (in SVMEA) thermotolerance is associated with high antioxidant enzyme levels while the reverse is true in the case of inherent (in SVMEA) and induced (in MEA) thermosensitivity. These findings suggest that increased production of oxygen free radicals may be involved in hyperthermic cell injury, which then becomes a function of basal or inducible levels of antioxidant enzymes. Induction of the latter by hyperthermia is apparently inefficient in transformed cells making them more vulnerable. Enzyme induction seems also to require a lag period of 4–6 h suggesting the possible involvement of an intermediate inducer(s) at molecular level. The so-called heat shock proteins may be candidates for such a role.

INTRODUCTION

The biological effects of hyperthermia (41–45°C) on mammalian cells have been studied extensively due to its potential role as a selective agent for cancer therapy (1). Numerous *in vitro* and *in vivo* studies indicate that neoplastic cells are more thermosensitive than normal cells (2–6) although exceptions and variations in thermal response of tumors do exist (7, 8). The exact mechanism(s) by which heat kills cells, however, remains unknown although cellular membranes, cytoskeletal structures, energy (respiratory) metabolism, protein synthesis, or DNA may be targets for hyperthermic injury (3, 9–12). Nevertheless, accumulating evidence indicates that hyperthermia may act as a form of oxidative stress. For instance, hyperthermia potentiates the cytotoxic effect of X-irradiation and other free radical generating antineoplastic agents such as the antibiotics Adriamycin and bleomycin (2, 13). Exposure to hyperthermia is associated with induced or enhanced synthesis of a family of highly conserved polypeptides known as heat shock proteins which are also induced by a variety of oxidizing agents (14, 15) and upon reoxygenation after prolonged anoxia (16–18). Furthermore, hyperthermia and various HSP[2] inducers have been shown to cause the accumulation of polyadenylated nucleotides in treated bacteria (19), and it has been proposed that one or more of these nucleotides may serve as a common mediator (alarmone) in cellular response to oxidative stress.

Since the common mechanism by which many oxidizing agents are known to cause cell injury is the increased production of oxygen free radicals, it follows that hyperthermia may have a similar pathogenetic mechanism. Increased thermal sensitivity of transformed cells, then, may be related to their low rate of free radical removal by the various free radical scavenging systems. This may indeed be the case since many neoplastic (20, 21) and established (immortal or weakly transformed) (22) cell populations were found to have low levels of SOD activity. Two forms of the latter are known in advanced organisms; one is a primarily cytosolic CuZn-SOD and the other is a primarily mitochondrial Mn-SOD. Both protect cells from the harmful superoxide radical ($O_2^{\cdot-}$) by converting it to hydrogen peroxide which is then metabolized by catalase and GSH-Px (23). Conversely, thermotolerant cells were shown to have relatively higher levels of SOD and certain low molecular weight reductants such as glutathione (24).

In order to study the role of free radical metabolism in normal and transformed cell response to hyperthermia, we used our previously characterized system (6, 25) of cloned normal (early passage, unestablished, or mortal) mouse embryo cells and their SV40-transformed derivatives to quantitate cell survival and the activities of major antioxidant enzymes (SOD, catalase, and GSH-Px) upon exposure to lethal or sublethal (tolerance-inducing) hyperthermia and in the presence of diethyldithiocarbamate, an inhibitor of CuZn-SOD. This system offers the advantage of dealing with relatively homogeneous (cloned) normal and transformed cell populations that are genetically related, so that any observed differences are more likely to be products of the transformation process rather than inherent tissue variations.

MATERIALS AND METHODS

Normal and transformed cells used in this study were the same clonal populations that we previously utilized for hyperthermia-related studies (6). Frozen stock cultures were thawed as needed and maintained at 37°C and 10% CO_2 in Dulbecco's modification of Eagle's minimum essential medium supplemented with 10% fetal bovine serum. Monolayers were seeded at a density of 5×10^4 cells/cm^2 and maintained in the above medium for 48 h before subjecting them, without further medium change, to the various treatments. Exposure to hyperthermia as well as determination of relative survival (relative plating efficiency) were done as described previously (6). For a lethal hyperthermic effect cells were exposed to 44°C for 1–3 h. Sublethal treatment and testing for thermotolerance was done by exposing cells to 45°C for 15 min, followed by incubation at 37°C for 2–6 h and subsequent challenge

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[2] The abbreviations used are: HSP, heat shock protein; SOD, superoxide dismutase; CuZn-SOD, copper- and zinc-containing SOD; Mn-SOD, manganese-containing SOD; DDTC, diethyldithiocarbamate; PBS, phosphate-buffered saline; GSH-Px, glutathione peroxidase; MEA, normal mouse embryo cells; SVMEA, SV40-transformed mouse embryo cells.

with heating for 1–4 h at 45°C. DDTC (Sigma Chemical Co.) treatment was done by incubating cells in culture medium containing 0–3 mM concentrations of the drug for 90 min at 37°C followed by washing 3 times with PBS and subsequent exposure to 37°C or 44°C for 1 h while in PBS. Plating efficiency was determined by plating a known number of cells and counting the number of colonies that developed in 2–3 weeks/100 cells plated. Cell survival and activities of SOD, catalase, and GSH-Px were determined in replicate cultures following each treatment.

For enzyme studies, subconfluent monolayers in 60-mm tissue culture dishes were washed 3 times with PBS and cells were scraped off in 1 ml of 50 mM phosphate buffer (pH 7.8) with a rubber scraper. Aliquots of cells were taken for cell counting in a hemocytometer and the remainder were lysed at 4°C by pulse-sonication (Heat System W-375 Sonicator) at 50% power for 2 min. Sample aliquots were used for catalase and GSH-Px assays, either immediately or within 3 days of storage at 4°C. For SOD activity, the remainder of the sample was either assayed immediately or stored at −20°C and assayed later. In either case, before the actual assay, low molecular weight reductants were removed by repeated concentration and buffer reconstitution using Amicon-YM10 microconcentrators.

Catalase was assayed by monitoring the change in absorbance of hydrogen peroxide at 240 nm according to the method of Aebi (26). One unit of activity is defined as 1 μM H_2O_2 degraded per min at pH 7.0 using an extinction coefficient of 43.6 mol^{-1} cm^{-1} (27).

GSH-Px activity was measured as described by Flohe and Gunzler (28) by coupling the reaction of GSH-Px and *t*-butylhydroperoxide with GSH reduction and NADPH oxidation by GSH reductase. One unit is the equivalent of 1 μM NADPH oxidized per min at pH 7.0 as measured by monitoring absorbance at 340 nm, with an extinction coefficient of 6.2×10^3 mol^{-1} cm^{-1}.

SOD activity was assayed at pH 7.8 by measuring the inhibition of cytochrome *c* (10 μM) reduction caused by superoxide ions generated by xanthine (0.10 mM)-xanthine oxidase reaction. Concentration of xanthine oxidase was adjusted to cause a reduction of cytochrome *c* at the rate of 0.025 absorbance unit/min at 550 nm (29). One unit is defined as 50% inhibition of the standard rate of reduction of cytochrome *c* (30). The assay mixture contained 10 μM KCN to inhibit endogenous cytochrome oxidase activity. Each sample was assayed twice for SOD activity, once to measure total SOD activity, and a second time in the presence of 5 mM KCN to inhibit CuZn-SOD and thus measure Mn-SOD activity alone. Boiling samples prior to the assay abolished all enzymatic activity. All enzyme activities were standardized to units/10^7 cells. The Student *t* test was used to compare experimental and control values and to calculate the level of significance for any observed differences.

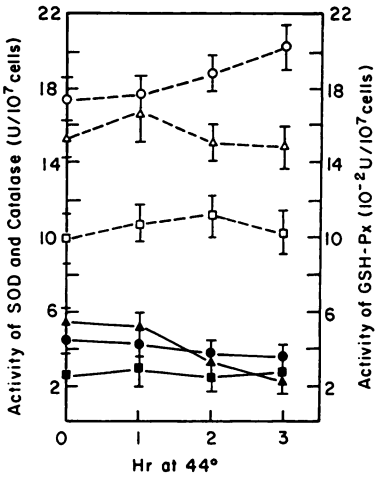

Fig. 1. Effect of lethal hyperthermia on antioxidant enzyme levels in MEA (- - -) and SVMEA (——) cells. Subconfluent monolayer cultures were exposed to 44°C for 0–3 h, after which they were washed with PBS and cells from each dish were scraped off in 1 ml of 50 mM phosphate buffer, pH 7.8. Aliquots were taken for cell counting and the remainder were processed for enzyme activity determination as described in "Materials and Methods." ○, ●, SOD; △, ▲, catalase; □, ■, GSH-Px. *Points*, means of values obtained from six replicate dishes; *bars*, SE.

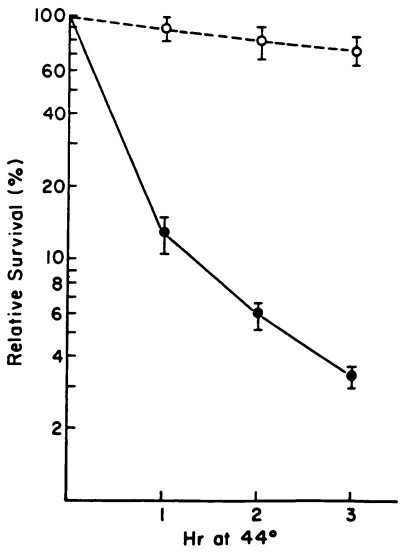

Fig. 2. Effect of hyperthermia on relative survival of MEA (○) and SVMEA (●). Monolayers were exposed to 44°C for 0–3 h, after which they were trypsinized and replated at sparse densities. Plating efficiency was determined by counting colonies that developed in 2–3 weeks/100 cells plated. Relative survival is expressed as percentage of plating efficiency of heated *versus* unheated controls (0 h at 44°C). *Points*, means from six replicates; *bars*, SE.

RESULTS

Presented in Figs. 1–6 are the results of cell survival and enzyme activity measurements. Fig. 1 shows that at 37°C, activity of all three enzymes (SOD, catalase, and GSH-Px) was 2–5 times higher in the normal than in the transformed cells (significant difference, $P < 0.01$). The relatively lowest activity in SVMEA was that of GSH-Px. Mn-SOD activity was extremely low in MEA and almost undetectable in SVMEA so that only total SOD (mostly CuZn-SOD) activity is presented here. More than 1 h heating at 44°C (or 45°C; data not shown) did not significantly alter enzyme levels in either cell population except for a slight reduction of SOD and catalase activities which was statistically significant ($P < 0.05$) in the case of catalase from the transformed cells only, after 3 h of heating. In contrast to its effect on enzyme activities, hyperthermia selectively reduced transformed cell survival (Fig. 2) to less than 5% compared to a normal cell survival of about 75% after 3 h at 44°C. Preconditioning cells by a brief, sublethal heating (15 min at 45°C) and reincubation at 37°C for 6 h had two significant effects: activities of SOD ($P < 0.01$) and GSH-Px ($P < 0.05$) almost doubled (Fig. 3); and cells acquired a significant ($P < 0.01$), relative thermal resistance against a second heat challenge of 1–4 h at 45°C (Fig. 4). The increments in both parameters, however, were far short of bringing the levels of the transformed cells to those of their normal counterparts.

Treatment with DDTC caused up to 80% inhibition of SOD activity in both MEA and SVMEA (Fig. 5) but had no effect on either catalase or GSH-Px. Exposure of cells to DDTC alone (at 37°C) was associated with moderate, but selective killing of transformed cells, reducing their relative survival to about 55% at 3 mM drug concentration. Survival of normal cells was only slightly affected (Fig. 6). Combined exposure of cells to DDTC and hyperthermia (1 h at 44°C) resulted in a significant killing of normal cells which, at 3 mM DDTC, reduced their survival to about 25%. The combined treatment was extremely destructive to the transformed cells at much lower DDTC concentrations, and above 1.5 mM it was associ-

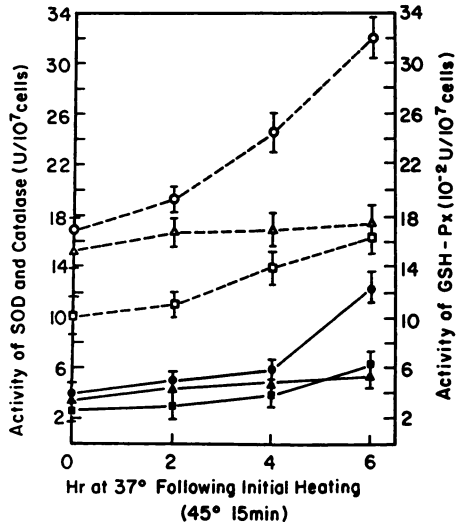

Fig. 3. Effect of sublethal hyperthermia on antioxidant enzyme activities of MEA and SVMEA. Monolayers were first exposed to 44°C for 15 min, followed by reincubation at 37°C for 0, 2, 4, and 6 h. At each *time point*, enzyme activities were determined in replicate cultures. Methodology and symbols as in Fig. 1.

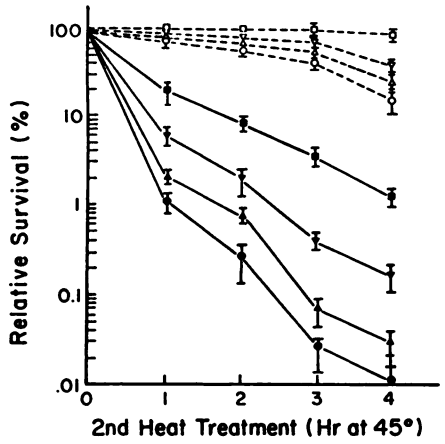

Fig. 4. Induction of thermotolerance after initial sublethal heating. Monolayers of MEA (- - -) and SVMEA (——) cells were first exposed to 45°C for 15 min, followed by incubation at 37°C for 0 h (○, ●), 2 h (△, ▲), 4 h (▽, ▼), and 6 h (□, ■) before a second heat challenge at 45°C for 0–4 h. Relative survival was determined after each *point* as described in Fig. 2.

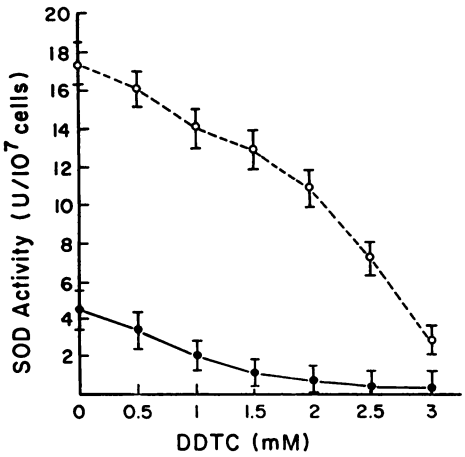

Fig. 5. Effect of DDTC treatment on SOD activity in MEA (○) and SVMEA (●). Cells were incubated in culture medium containing 0–3 mM DDTC for 90 min at 37°C followed by washing 3 times with PBS and harvesting cells for enzyme activity assay as described in Fig. 1. *Points*, means of six replicates; *bars*, SE.

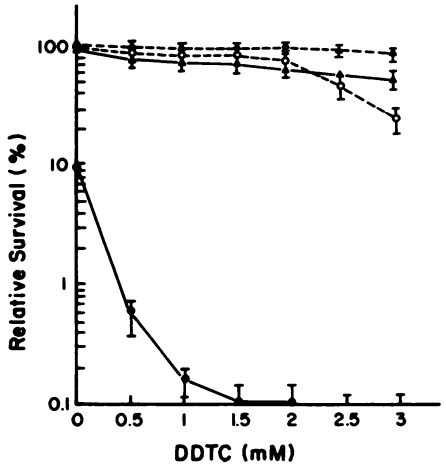

Fig. 6. Effect of pretreatment with DDTC on cell killing by hyperthermia. MEA (- - -) and SVMEA (——) were first treated with DDTC as described in Fig. 5, which was followed by either continued incubation at 37°C (△, ▲) or exposure to 44°C for 1 h (○, ●). Survival was determined as described in previous figures.

ated with instantaneous cell killing and detachment of monolayers, thus hampering accurate quantitation of survival at these concentrations.

DISCUSSION

We have shown that transformed cells possess markedly low levels of antioxidant enzyme activities. These cells were selectively killed by exposure to lethal hyperthermia. Normal cells contained significantly higher enzyme activities and were remarkably resistant to hyperthermic injury. Pharmacologically lowering the activity of CuZn-SOD in normal cells, via DDTC inhibition, led to a marked reduction in their thermal resistance. Other investigators (31, 32) have also shown pretreatment with DDTC to potentiate radio- and thermosensitivity of Chinese hamster cells. DDTC is a copper-chelating agent which in addition to CuZn-SOD can also inhibit other copper-containing enzymes (33) such as cytochrome oxidase, a respiratory chain enzyme (34) and an O_2^- scavenger (35). Thus, the cumulative effect of DDTC treatment is a substantial increase in superoxide levels.

These findings suggest that oxygen free radicals and cellular antioxidant defenses may, indeed, play an important role in the pathogenesis of hyperthermic injury. The natural thermosensitivity of SVMEA may be a direct result of their low antioxidant enzyme activities and hence higher levels of oxygen free radicals, which accumulate and remain unmetabolized upon hyperthermic exposure. Apparently, low levels of SOD activity, such as those of untreated SVMEA or DDTC-treated MEA, are sufficient for cell protection under conditions of normal metabolism (at 37°C). Lowering SOD activity below that point, however (DDTC treatment of SVMEA or combined DDTC-heat treatment of MEA), may leave the cells profoundly vulnerable to free radical injury.

The reduction in the catalase activity of the transformed cells after prolonged heating is probably a result of inactivation by higher O_2^- levels (36), since the catalase of normal cells was not affected; that is, assuming structural similarity.

Preconditioning cells with sublethal hyperthermia resulted in an increase in their antioxidant enzyme activities, which was associated with the induction of a relative thermotolerance. Loven *et al.* (37) using a similar scheme of fractionated hyperthermia, also documented an increase in CuZn-SOD activity in

Chinese hamster ovary and ovarian carcinoma cells. These data, once again, suggest the possible involvement of free radical metabolism in cellular response to hyperthermia.

The molecular mechanism by which sublethal hyperthermia may cause an induction of higher enzyme activity is not clear. The fact, however, that it took several hours for this induction to reach a significant level suggests the possible involvement of an "intermediate" messenger molecule(s) that hyperthermia would induce first. HSPs (or one of them) are potential candidates for such a role since their synthesis is usually induced within a few minutes of hyperthermic exposure (38). Enhanced synthesis of HSPs is known to be associated with the appearance of thermotolerance in sublethally heated cells and cross-tolerance can be induced by other HSP inducers (13, 16, 39), some of which are also known inducers of SOD (40, 41).

During the described "lag" period before antioxidant enzymes reach "thermoprotective" levels, cells apparently can rely only on their preexisting enzyme levels. These levels are very low in transformed cells which may explain their selective destruction upon exposure to lethal temperatures. We have shown previously (6) that SVMEA cells actually contain higher than normal levels of basal (constitutive) and inducible HSPs, which is contradictory to the presumed thermoprotective role of these proteins. A possible explanation for this seeming paradox is that the hypothesized HSP-enzyme connection is defective or inefficient in transformed cells. Such metabolic defects are not peculiar to the transformed phenotype. We are currently investigating the outlined hypotheses.

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