Clonogenicity of human leukemic cells protected from cell-lethal agents by heat shock protein 70

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Abstract Pretreatment of human leukemia THP-1 cells with heat shock protein Hsp70 (Hsp70) protected them from the cell-lethal effects of the topoisomerase II inhibitor, lucanthone and from ionizing radiation. Cell viability was scored in clonogenic assays of single cells grown in liquid medium containing 0.5% methyl cellulose. Colonies were observed and rapidly scored after staining with the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide. The frequency of abasic sites in the deoxyribonucleic acid (DNA) of THP-1 cells was reduced when these cells were treated with Hsp70. Hsp70 is presumed to have protected the cells by promoting repair of cell DNA, in agreement with previous studies that showed that Hsp70 enhanced base excision repair by purified enzymes. The shoulders of radiation dose-response curves were enhanced by pretreatment of cells with Hsp70 and, importantly, were reduced when cells were transfected with ribonucleic acid designed to silence Hsp70. Hsp70 influenced repair of sublethal damage after radiation.

INTRODUCTION

Protection of cells from heat shock, ionizing radiation, and other cell stress agents is mediated by the highly conserved family of heat shock proteins (Hsps), and the stress-inducible Hsp70B protein is especially important. Hereafter, Hsp70B is referred to as Hsp70 (for reviews see Georgopoulos and Welch 1993; Bukau and Horwich 1998; Morimoto 2002; Nollen and Morimoto 2002; Mosser et al 1997).

Deoxyribonucleic acid (DNA) damage is an important result of cell stresses, especially those induced by reactive oxygen species (ROS), but repair of specific damage in cellular DNA mediated by Hsps has not been extensively studied. This is surprising, in view of the numerous descriptions of cell DNA damage and the numerous suggestions that Hsp70 has a role in repair of this kind of stress damage.

Abundant evidence suggested that DNA repair might be involved in the cell stress response, and base excision repair (BER) appeared to be involved. For example, induced radioresistance by a member of the Hsp70 family

by low-dose radiation (Sadekova et al 1997), resistance to streptozotocin and reactive oxygen intermediates by heat shock in rat pancreatic islet cells (Bellmann et al 1995), and the natural resistance of human beta cells toward nitric oxide afforded by Hsp70 (Burkart et al 2002), all suggest a role for Hsps in cellular protection, through DNA repair. Mitochondria are protected by Hsps against oxidative injury (Polla et al 1996), and overexpression of inducible Hsp70 in mice afforded protection against neurodegeneration (Cummings et al 2001). Overexpression of Hsp70 also conferred protection against oxidative injury in HEPG2 cells (Hosoi et al 2002).

An interesting protective effect of Hsp70 chaperone function was found in SCA1 mice. Usually they develop spinocerebellar ataxia type 1, but crossbreeding with Hsp70 overproducer mice afforded progeny with protection against neurodegeneration. High levels of Hsp70 were not deleterious (Cummings et al 2001). ROS are believed to be the active agents in certain diseases of neurodegeneration. Therefore, some of the protective effects of Hsps are likely to reflect enhanced repair of oxidative damage to DNA of the genome and mitochondria. Hsps might be important players in cellular BER by stimulating the relevant enzymes that are involved in repair of cell DNA damage.

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Abasic sites

Although transient abasic sites are naturally occurring, their frequency can be greatly increased by a variety of genotoxic agents. Abasic sites would be hazardous for cell viability unless they were repaired promptly because abasic sites could lead to double-strand breaks if cellular topoisomerase II were to encounter them (Kingma et al 1995). An essential next step in the BER pathway to eliminate abasic sites is the 5' incision of the abasic site on the damaged strand. Human apurinic/apyrimidinic (AP) endonuclease (HAP1, also described as APE1/REF1) performs this single-strand scission, leaving the requisite 3' OH on the adjacent nucleotide, a requirement for the next step in repair synthesis by DNA polymerase β (β pol). A potentially obstructive 5' sugar remnant, which has aldehyde properties, is removed by a DNA deoxyribosephosphodiesterase function provided by a portion of the DNA polymerase β (β pol) (Matsumoto and Kim 1995). DNA polymerase β (β pol) then fills in the single-strand gap, which is ultimately sealed by DNA ligases.

Hsp70 in vitro stimulated the first step in BER by uracil DNA glycosylase (unpublished data), the HAP1 step was stimulated 10- to 100-fold by Hsp70 (Kenny et al 2001), and the 43- to 48-kDa N-terminal adenosine triphosphate (ATP)-binding portion of Hsp70 was responsible for this activity (Mendez et al 2003b). Hsp70 also stimulated repair by β pol 5- to 10-fold (Mendez et al 2003a).

Another enzyme, DNA topoisomerase 1, was reactivated by Hsc70. It mediated an ATP-independent mechanism in vitro and in cells (Ciavarrá et al 1994). A subpopulation of Hsp70 in mitochondria of *Saccharomyces cerevisiae* interacted with endonuclease Sca I, causing a change in enzyme specificity; interestingly, the N-terminal adenosine triphosphatase domain and not its unfolded substrate binding domain was involved (Mizumura et al 1999). Other interactions of DNA repair enzymes and Hsps are only now beginning to be recognized.

Stress-induced apoptosis is influenced by Hsp70

The chaperone function of Hsp70 is required for protection against stress-induced apoptosis (Mosser et al 2000). Elevated levels of the full-length Hsp70 were required for inhibition of cleavage of the caspase 3 substrate. Overexpression of Hsp70 and Hsc70 (constitutive Hsp70) prevented processing of procaspase 3 and 9, thereby preventing apoptosis. Hsp70 itself does not inhibit caspase 3 in vitro. Repression of the Hsp70 promoter by NFIL6, Ku70, and mitogen-activated protein kinase involves 3 complementary mechanisms, which ensure the timely return of Hsp70 to baseline levels (Tang et al 2000).

Interestingly, the selective depletion of Hsp70 after induction or transfection with antisense Hsp70 DNA

caused massive apoptosis-like cell death in tumor cell lines. Nonmalignant cells were refractory to this treatment (Nylandsted et al 2000). The mechanism is not understood, but the tumor-specific death program due to deficiency of Hsp70 is independent of caspases and it bypasses Bcl-2. A strategy to treat cancer using antisense Hsp70 DNA was advanced by demonstrating eradication of xenografts in immune-deficient mice (Nylandsted et al 2002). Hsp70 may inhibit an as yet unknown apoptotic pathway.

Parenteral administration of Hsp70 in animals or humans might be feasible because it is now "clear that Hsps can and do exit mammalian cells, interact with cells of the immune system and exert immunoregulatory effects" (Asea et al 2002). Fujihara and Nadler (1999) showed that exogenous Hsp70s enter certain cells and transport the proteins to nuclei. Schemes to exploit BER enhancement would not be difficult to imagine.

Previous publications demonstrated specific attachment and strong stimulation of DNA BER enzymes by Hsp70 in vitro (Mendez et al 2000, 2003a, 2003b; Kenny et al 2001). We reasoned that cell viability (clonogenic fraction) might be protected by the enhanced BER to be expected in Hsp70-treated cells. This could be the result of direct attachment of Hsp70 to the BER enzymes or to stimulation of transcription (Asea et al 2002). Obstacles to studying clonogenicity after Hsp70 treatment had to first be overcome.

Colony formation in vitro has long been studied with cells that grow attached to glass or plastic surfaces, but Hsp70 enters these cells poorly, if at all. Cells of hemopoietic origin take up Hsp70 avidly but do not attach to surfaces; therefore, their clonogenic ability is not easily measured. We overcame these problems, as described below. Here, THP-1 acute monocytic leukemia cell lines were studied because they take up exogenous Hsp70 from their culture medium. A novel single-cell survival test had to be developed because THP-1 cells grow only in suspension. Single cells were cultured in 0.5% methyl cellulose for 14–21 days; their clones were then stained for counting by 30 minutes exposure to a tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide [MTT]).

Exposure to lucanthone increased the frequency of abasic sites in human HeLa cells (Mendez et al 2002) and in THP-1 cells (unpublished data). Cells grown in lucanthone exhibited progressive loss of colony-forming ability, but loss of cell viability was prevented by pretreatment with Hsp70. Pretreatment also enhanced the shoulder region of radiation dose-response curves.

MATERIALS AND METHODS

The THP-1 acute monocytic leukemia cell line was purchased from the American Type Culture Collection

(ATCC catalog no. TIB-202, Manassas, VA 20108, USA). Cells grew in modified Roswell Park Memorial Institute (RPMI) medium 1640 with 10% fetal bovine serum in a humidified incubator at 37° C, with 5% CO₂ in air; the doubling time for the cells in culture was 36 hours. Hsp70 was added to 1 or 2 µg/mL for at least 3 hours. Hsp70 was purchased from StressGen, Victoria, British Columbia, Canada.

THP-1 colony-forming assay

Cells in suspension culture were dispersed by pipetting to provide single-cell suspensions. Cells were incubated at 10⁴ cells/mL in plastic tubes in a CO₂ incubator for 3 hours with or without Hsp70. Cells were then resuspended and incubated for 14–21 days in growth medium containing 0.5% methyl cellulose with care not to move the 60-mm petri dishes, thereby avoiding interference from secondary colonies. Methyl cellulose inhibited cells from dispersing from the growing colonies (Sigma, MO 512, 88,000, St. Louis, MO 63108, USA).

Colonies were counted under 10 power magnification with a dissecting microscope after 30 minute exposure to the hydrogen acceptor, MTT (Sigma, M 2128), in a final concentration of 0.4 mg/mL for 30 minutes at 37° C. Cells grown in medium with high glucose concentration exhibit especially strong dark brown purple formazan staining (Vistica et al 1991). Colonies contained >500 cells by 14 days. Abortive cell colonies containing fewer than 32 cells were ignored.

Lucanthone hydrochloride

Lucanthone hydrochloride (R009CJ) was a gift from Sterling Winthrop Research Institute, Rensselaer, NY; it was 97.5% pure by high-performance liquid chromatography mass spectroscopy. Lucanthone's lethal effects in HeLa cells were rapidly reversed by timely dilution of the cells, thereby reducing lucanthone concentration in single-cell assays (Bases 1970).

Ionizing radiation

Cells were exposed to gamma radiation from a ¹³⁷Cs source at 7.89 Gy/min.

Aldehyde-reactive probe and slot-blot

The procedures described by Nakamura and Swenberg were followed, with minor modifications, using aldehyde-reactive probe (ARP) and slot-blot methods. The ARP contains a biotin moiety destined to react with streptavidin-conjugated horseradish peroxidase. Fifteen micrograms of THP-1 cell DNA in 150 µL of phosphate-

buffered saline was incubated with 1 mM ARP (Dojindo Laboratories, Gaithersburg, MD, USA) at 37° C for 10 minutes. The DNA was precipitated in 95% ethanol with 0.3 M sodium acetate, pH 7.0. After rinses in 70% ethanol and 95% ethanol, the labeled DNA was dried, dissolved in distilled water, and the DNA concentration was determined by OD₂₆₀.

After heat denaturation at 100° C for 5 minutes, DNA in 200 µL of water was quickly cooled on ice and precipitated with an equal volume of 2 M ammonium acetate. Aliquots of 1, 0.5, and 0.1 µg were bound to a Hybond-C Super membrane (Amersham, Piscataway, NJ, USA). After soaking in 5× standard saline citrate for 15 minutes at 37° C, the membrane was baked in a vacuum oven at 80° C for 1 hour, and nonspecific proteins were blocked by preincubation of the membrane with 5% nonfat milk in phosphate-buffered saline with 0.05% Tween 20 (PBST) at 4° C overnight. The membrane was incubated in PBST with streptavidin-conjugated horseradish peroxidase (Bio-Genex, San Ramon, CA, USA) at room temperature for 40 minutes. Finally, the membrane was rinsed in PBST and the peroxidase activity observed by enhanced chemiluminescence (Amersham). The image was developed by exposing Fuji medical X-ray film for 1 second–2 minutes; it was scanned and analyzed with a Molecular Dynamics densitometer (Sunnyvale, CA, USA) using ImageQuant software. Peroxidase-generated X-ray film densities obtained in slot-blot determinations of abasic sites in cellular DNA were compared with the densities obtained from corresponding amounts of pBR322 DNA, which had been prepared to contain 1 abasic site/plasmid. The ARP reactivity in native pBR322 was 6-fold lower. Depurinated pBR322 preparations containing ~1 abasic site/plasmid were used as reference standards to determine the frequency of abasic sites in THP-1 cell DNA in comparative ARP slot-blot assays.

Interfering RNA

A pool of duplex small interfering duplex ribonucleic acid (RNA) designed to silence HSP1b on transfection into cells (Dharmacon M-003501-00, Lafayette, CO 80026, USA) was used in conjunction with oligofectamine, Invitrogen (catalog no. 12252-011, Carlsbad, CA 92008).

Fifty µL of 20 µM oligonucleotide was added to 800 µL of RPMI 1640 medium without serum; 40 µL of oligofectamine was first added to 110 µL of RPMI 1640. On mixing with the oligonucleotides, conjugates formed, as described by the manufacturer. The mixture was then diluted to a total of 5.0 mL in RPMI 1640 without serum, and transfection of 15 × 10⁶ cells in suspension took place for 2 hours at 37° C in a CO₂ incubator. Cells were then diluted to 30 mL in RPMI 1640 with 10% fetal bovine serum, for growth overnight at 37° C in the CO₂ incubator.

Fig 1. Frequency of apurinic/aprimidinic (AP) sites in THP-1 cell deoxyribonucleic acid (DNA) after exposure to Hsp70 in growth medium. DNA was purified and the reduction in AP site frequency was scored by the ARP reaction in slot-blot determinations in 3 separate experiments as described in Materials and Methods. Untreated cells had 3.9 AP sites/ 10^6 nucleotides.

Cells were harvested for irradiation and assays as described previously.

RESULTS

Hsp70 lowers the frequency of AP sites in THP-1 cell DNA

The frequency of abasic sites in THP-1 cells was determined by the ARP method (Mendez et al 2002). The value of 3.9 ± 0.7 SE sites/ 10^6 nucleotides for THP-1 cells was in general agreement with our previous values of 9.9 ± 2.8 SE AP sites/ 10^6 nucleotides obtained for HeLa cells and 9 AP sites/ 10^6 nucleotides for human liver cells (Nakamura et al 1998) and 9.1 AP sites/ 10^6 nucleotides in human lung cells (Atamna et al 2000). SE is the standard error of the mean.

Results of Asea et al 2002 showed that optimum stimulation of transcription in THP-1 cells could be achieved by several hours exposure to 1 $\mu\text{g/ml}$ of exogenous Hsp70. Reduction in AP sites, enhancement of clonogenicity after lucanthone treatment, and enhancement of the shoulders in radiation dose-response curves were studied in THP-1 cells pretreated with 1 $\mu\text{g/ml}$ of Hsp70.

After 3 or 4 hours of exposure to 1 $\mu\text{g/ml}$ of recombinant Hsp70, the frequency of AP sites in THP-1 cells was reduced by half in the 3 independent experiments shown in Figure 1. Lower concentrations of Hsp70 failed to significantly achieve this reduction.

The reduction in AP sites induced by 1 $\mu\text{g/ml}$ of Hsp70 in the 3 experiments of Figure 1 is significant at $P < 0.10 > 0.05$. Nonspecific effects are unlikely to account for the results, which were achieved by addition of 1 $\mu\text{g/ml}$ Hsp70 to cells growing in 10% fetal bovine se-

rum (ie, in 10^5 -fold greater concentration of serum proteins). Because 48- and 43-kDa fragments of Hsp70 share the native molecule's ability to enhance BER enzyme activity, ambiguities inherent in experiments with denatured Hsp70 were avoided (Mendez et al 2003b).

Pretreatment of THP-1 cells with 40 μM lucanthone increased the frequency of AP sites in the DNA of the lucanthone-treated cells to 108% of control values, but pretreatment of cells with 1 $\mu\text{g/ml}$ of Hsp70 reduced the AP frequency to 34% of control values; without lucanthone, Hsp70 treatment reduced AP site frequency to 41% (not shown). This was expected from results shown in Figure 1. The assay of AP sites with and without lucanthone treatment would be identical under conditions of the ARP probe. [With HeLa cells, 8 μM and 80 μM lucanthone was more active; it increased the frequency of AP sites 50% and 300% (Mendez et al 2002)].

Colony formation of THP-1 cells in 0.5% methyl cellulose

THP-1 cells were incubated in 60-mm petri dishes for 14–21 days. Figure 2 shows their appearance under phase contrast (Fig 2A, left photo). Purple formazan polymer was deposited in the cells after 30 minutes at 37° C in 0.4 mg/mL of MTT for ease of counting (Fig 2B, right panel). The appearance of a petri dish with MTT-stained THP-1 cells at 10-fold magnification is shown in Figure 2C. The efficiency of colony formation was 5–10%. Figure 3 shows that the number of colonies determined was linearly related to the initial cell inoculum; linearity was obtained with lower plating efficiencies also.

Fig 2. THP-1 colonies grown from single cells in modified Roswell Park Memorial Institute (RPMI) medium 1640 with 10% fetal bovine serum in 0.5% methyl cellulose. (A) A colony of THP-1 cells by phase contrast microscopy. (B) A THP-1 cell colony after staining with 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT). (C) Part of a petri dish containing stained colonies. The scale bars indicate magnification. For (A) and (B) the bar represents 50 μ m; for (C) it represents 0.5 cm.

Fig 3. Colony plating efficiency of THP-1 cells in 0.5% methyl cellulose-containing medium in 60-mm petri dishes. The plating efficiency was 5.7%.

Hsp70 protects cell viability of THP-1 cells in 80 μ M lucanthone

Loss of colony formation was related to the length of time cells were exposed to lucanthone before rescue by dilution in assay plates. During several months of continuous culture, THP-1 cells became somewhat less sensitive to

exposure to 80 μ M lucanthone. Nevertheless, in all 3 consecutive experiments shown in Figure 4, pretreatment of cells for 3 hours with 1 or 2 μ g/mL Hsp70 provided highly significant protection of cell viability ($P < 0.01$). The effects of Hsp70 in these preliminary experiments do not exclude other mechanisms than ARP removal for Hsp70 protective effects.

Hsp70 protects cell viability of irradiated THP-1 cells

We found that after 2.63 Gy, THP-1 cell survival relative to control values was reduced to 0.35 ± 0.04 SE but with pretreatment by Hsp70, survival was greater, 0.60 ± 0.04 SE ($P < 0.01$) (Fig 5A). Figure 5B shows similar results in a radiation dose-response experiment with THP-1 cells 3 hours after exposure to 2 μ g/mL of Hsp70.

Figure 5A,B shows that Hsp70 pretreatment of THP-1 cells increased the width of the shoulder of their radiation dose-response survival curves. Their D_0 , the dose required to reduce cell survival to $1/e$ or 37% on the straight line portion of the curve, was 1.0 Gy with or without Hsp70 pretreatment. The shoulder is considered to reflect repair of sublethal damage, as studied in split-dose radiation treatments by many authors.

Transfection of cells with small interfering RNA to silence Hsp70 abolished the shoulder of radiation dose-response curves

Transfection of THP-1 cells with a pool of double-stranded RNA that had been designed to silence Hsp70-2 was done as described in Materials and Methods. Transfection

Fig 4. Hsp70 pretreatment at 1 μ g/mL Hsp70 for 3 hours protected THP-1 cells from sterilization by later exposure to 80 μ M lucanthone. Protection of colony formation in each of the 3 experiments shown was significant at $P < 0.01$, after 1 hour or 2.5 hours of exposure to lucanthone. Symbols bearing error bars signify standard errors of means. Open symbols, Hsp70 pretreatment for 3 hours before challenge; closed symbols: no Hsp70 pretreatment. Cells were less sensitive to lucanthone after 3 months of continuous culture.

of cells with a nonspecific RNA (scrambled sequence) served as a control. Twenty-four hours after transfection and growth in culture, the cells were challenged with ^{137}Cs γ irradiation and their colony-forming fractions determined, as described previously.

Figure 6 shows that the cells transfected with the interfering RNA exhibited little or no shoulder in their dose-response curve. Cells transfected with the nonspecific RNA clearly retained the usual shoulder.

DISCUSSION

These results clearly demonstrate that pretreatment of THP-1 cells with Hsp70 established resistance to lucanthone's and radiation's lethal effects. Stimulation of HAP1 and β pol to eliminate abasic sites might well account for this protective effect, but other mechanisms of Hsp70 stimulation are still not excluded. Repair of DNA damage in THP-1 cells after ionizing radiation was also stimulated by Hsp70, presumably after stimulation of β pol repair of strand breaks (Mendez et al 2000). Pretreatment of THP-1 cells by Hsp70 for 3 hours enhanced the size of

A HSP70 Enhanced Resistance of THP-1 Cells to Ionizing Radiation

B HSP70 Enhanced Resistance of THP-1 Cells to Ionizing Radiation

Fig 5. Radiation dose-response of THP-1 cells with $\square$ or without $\bullet$ pretreatment for 3 hours with 1 μ g/mL of Hsp70 (A) or with 2 μ g/mL of Hsp70 (B). Standard error terms are shown. In (B), after 2.63 Gy, the difference in survival was significant at $P < 0.05$. Survival of cells pretreated with Hsp70 and then given 7.89 Gy were not different from control values. Standard error terms were too large to be meaningful and are omitted for clarity in the insert that is from the same study. Plating efficiency for cells treated with Hsp70 in the experiments of (A) and (B) were 12% and 7.2%, respectively. Corresponding control plating efficiencies in the 2 experiments were 13% and 8.9%.

Fig 6. Transfection of THP-1 cells with RNA designed to silence Hsp70 reduced the shoulder of their radiation dose-response curve. Transfection was done as described in Materials and Methods. The clonogenic efficiencies of silenced and control cells were 10.4% and 11.6%, respectively. Their Do's were also unchanged, ie, 1.50 and 1.40. ●, radiation survival of cells 24 hours after transfection with control, scrambled RNA; △, radiation survival 24 hours after transfection with RNA designed to silence Hsp70.

the shoulder in dose-response curves (Fig 5). These results support the notion that Hsp treatment enhanced repair of sublethal radiation damage. The identity of the lesions involved is still not known. Interestingly, repair of potentially lethal radiation damage (as measured by Do) was not changed. Potentially lethal damage is exhibited when irradiated cells are treated with chemotherapeutic agents. Presumably, DNA is the major target, and the different kinds of damage are repaired by corresponding mechanisms. Hsp70 clearly enhanced repair, presumably by the enzymes of BER in cells cultured in 10% fetal bovine serum.

The low doses of ionizing radiation that institute the adaptive response, ie, enhanced resistance to subsequent radiation challenges, did not induce expression of BER enzymes or their messenger RNAs (mRNAs) in human lymphoblastoid cells (Inoue et al 2004). The mRNA and cellular BER protein levels were not elevated. Nevertheless, BER enzymes remain leading candidates for increased activity in the adaptive response. BER enzyme activities might instead be enhanced by induction of Hsps that, in turn, would be expected to increase BER

activity and their transport into cell nuclei, perhaps accounting for the paradox.

Entry of Hsp70 into THP-1 cells during a 3-hour exposure to this Hsp has been documented (Asea et al 2002). HeLa cells and cells of nonhemopoietic origin are generally refractory. Here, we showed that Hsp70 exposure of THP-1 cells led to reduction in the frequency of AP sites in their DNA. Presumably, HAP1 and β pol activities were stimulated in these cells, as expected from our previous findings (Kenny et al 2001; Mendez et al 2003a; 2003b). Protection of THP-1 clonogenicity by Hsp70 was clearly demonstrated (Fig 4). Protection of cell viability after ionizing radiation was also observed (Fig 5). The novel method for evaluating cell survival in suspended THP-1 cells may be useful in other studies. It measures cells' ability to continuously divide. Cells that escape apoptosis but are sterile or are capable only of abortive division would not be scored as viable by this method. Therefore, this method could supplement other kinds of viability assays and is perhaps more definitive.

Stimulation of DNA repair in cells by Hsps has been considered before as a mechanism to understand results in vitro and in clinical situations. Ciocca and collaborators have called attention to the condition of elevated Hsps in influencing chemotherapy outcomes (Nadin et al 2003). Elevated Hsp levels correlated with resistance to chemotherapy, presumably because of enhanced repair of DNA damage. Our results point to the possible mechanism at the level of cellular DNA repair of AP sites. Hsps could be expected to endow hemopoietic cells, which presumably do take up Hsps, with relative resistance to chemotherapy because of enhanced DNA repair. Epithelial cells, presumably less likely to take up Hsps, would be relatively sensitive to agents that damage DNA.

Cyclosporine is an immunosuppressant that protects organ grafts from rejection. It regulates stress-induced apoptosis, increases cardiomyocyte Hsp70, and may induce an Hsp protective effect (Chen et al 2002). Clinicians might enhance cellular Hsp70 levels and thereby promote BER by administering this widely used drug. Lucanthone's ability to prevent DNA repair of lethal cellular damage (Bases 1970) may be related to its action as a topoisomerase inhibitor (Bases and Mendez 1997). Accelerated regression of human tumors treated jointly with lucanthone and radiation (Del Rowe et al 1999) suggest a role for Hsp70 in influencing the response of tumor and adjacent normal tissues.

Results of Figure 5A,B showed that pretreatment of cells with exogenous Hsp70 increased their radiation resistance, apparently by enhancing DNA repair of sublethal radiation damage, as evidenced by the increased size of the shoulder of the dose-response curve. No significant change in the Do (the slopes of the curves) was found.

We reasoned that silencing of endogenous Hsp70

would be expected to abolish the shoulder but not the Do. Results of Figure 6 clearly confirmed this notion. After transfection with small interfering RNA designed to silence Hsp70, the shoulder was abolished but the Do was unchanged from the control value.

The results in this article support the notion that Hsp70 is important in enhancing repair of sublethal damage. Presumably, BER of DNA damage after ionizing radiation could account for this. Our previous publications described 10- to 100-fold enhanced BER repair enzyme activities by Hsp70. Other postradiation repair activities evidenced by the unchanged Do values do not appear to be greatly influenced by Hsp70.

RNA interference (reviewed in Novina and Sharp 2004) includes triggering digestion of specific mRNAs, but other silencing mechanisms could also account for the results in this study. Demonstration of reduced Hsp70 levels in cells after transfection with interfering RNA should be expected. Appropriate experiments based on data presented should now make it possible to identify the molecular correlates in DNA, which accompany the effects of Hsp70, described here.

Note in proof: Recent experiments showed that the frequency of abasic sites in DNA of THP 1 cells one day after siRNA transfection was higher than after transfection with scrambled RNA. After 0 Gy, 1.3 Gy, and 2.6 Gy, the abasic site frequencies in DNA of siRNA transfected cells were: 5.8-, 3.6-, and 1.7-fold higher than with scrambled RNA. Presumably, siRNA reduced the normal stimulus to base excision repair enzyme activity afforded by HSP 70, thereby leaving more unrepaid cell-lethal abasic sites. This might help account for the enhanced cell lethal effects found in the siRNA transfected cells after irradiation in the experiment of Fig. 6.

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REFERENCES

- Asea A, Rehli M, Kabingu E, Boch JA, Bare O, Auron PE, Stevenson MA, Calderwood SK. 2002. Novel signal transduction pathway utilized by extracellular Hsp 70. *J Biol Chem* 277: 15028–15034.
- Atamna H, Cheung I, Ames BN. 2000. A method for detecting abasic sites in living cells: age-dependent changes in base excision repair. *Proc Natl Acad Sci U S A* 97: 686–691.
- Bases R. 1970. Enhancement of X-ray damage in HeLa cells by exposure to lucanthone (Miracil D) following radiation. *Cancer Res* 30: 2007–2011.
- Bases R, Mendez F. 1997. Topoisomerase inhibition by lucanthone, an adjuvant in radiation therapy. *Int J Radiat Oncol Biol Phys* 37: 1133–1137.
- Bellmann K, Wenz A, Radons J, Burkart V, Kleemann R, Kolb H. 1995. Heat shock induces resistance in rat pancreatic islet cells against nitric oxide, oxygen radicals and streptozotocin toxicity in vitro. *J Clin Invest* 95: 2840–2845.
- Bukau B, Horwich AL. 1998. The Hsp 70 and Hsp 60 chaperone machines. *Cell* 92: 351–366.
- Burkart V, Liu H, Bellmann K, et al. 2002. Natural resistance of human beta cells toward nitric oxide is mediated by heat shock protein 70. *J Biol Chem* 275: 19521–19528.
- Chen H-W, Chien C-T, Yu S-L, Lee Y-T, Chen WJ. 2002. Cyclosporine A regulates oxidative stress-induced apoptosis in cardiomyocytes: mechanisms via ROS generation, iNOS and Hsp 70. *Br J Pharmacol* 137: 771–781.
- Ciavarrá RP, Goldman C, Wen K-K, Tedeschi B, Castora FJ. 1994. Heat stress induces hsc70/nuclear topoisomerase I complex formation *in vivo*. Evidence for hsc70-mediated, ATP-independent reactivation *in vitro*. *Proc Natl Acad Sci U S A* 91: 1751–1755.
- Cummings CJ, Sun Y, Opal P, Antalffy B, Mestril R, Orr HT, Dillmann WH, Zoghbi HY. 2001. Over-expression of inducible HSP70 chaperone suppresses neuropathology and improves motor function in SCA1 mice. *Hum Mol Genet* 10: 1511–1518.
- Del Rowe JD, Bello J, Mitnick R, et al. 1999. Accelerated regression of brain metastases in patients receiving whole brain radiation and the topoisomerase II inhibitor, lucanthone. *Int J Radiat Oncol Biol Phys* 43: 89–93.
- Fujihara SM, Nadler SG. 1999. Intranuclear targeted delivery of functional NF- κ B by 70 kDa heat shock protein. *EMBO J* 18: 411–419.
- Georgopoulos C, Welch WJ. 1993. Role of the major heat shock proteins as molecular chaperones. *Annu Rev Cell Biol* 9: 601–634.
- Hosoi N, Itoh H, Koyama K, Tashima Y. 2002. Overexpression of the heat shock protein 70 confers protection against oxidative injury in HEPG2 cells. *Transplant Proc* 34: 2647–2649.
- Inoue M, Shen G-P, Chaudhry MA, Galick H, Blaisdell JO, Wallace SS. 2004. Expression of the oxidative base excision repair enzymes is not induced in TK6 human lymphoblastoid cells after low doses of ionizing radiation. *Radiat Res* 161: 409–417.
- Kenny MK, Mendez F, Sandigursky M, Kureekattil RP, Goldman JD, Franklin WA, Bases R. 2001. Heat shock protein 70 binds to human apurinic/apyrimidinic endonuclease and stimulates endonuclease activity at abasic sites. *J Biol Chem* 276: 9532–9536.
- Kingma PS, Corbett AH, Burcham PC, Marnett LJ, Osheroff N. 1995. Abasic sites stimulate double-stranded DNA cleavage mediated by topoisomerase II. *J Biol Chem* 270: 21441–21444.
- Matsumoto Y, Kim K. 1995. Excision of deoxyribose phosphate residues by DNA polymerase β during DNA repair. *Science* 269: 699–702.
- Mendez F, Goldman J, Bases RE. 2002. Abasic sites in DNA of HeLa cells induced by lucanthone. *Cancer Invest* 20: 983–991.
- Mendez F, Kozin E, Bases R. 2003a. Heat shock protein 70 stimulation of the deoxyribonucleic acid base excision repair enzyme polymerase β . *Cell Stress Chaperones* 8: 153–161.
- Mendez F, Sandigursky M, Franklin WA, Kenny MK, Kureekattil R, Bases, R. 2000. Heat-shock proteins associated with base excision repair enzymes in HeLa cells. *Radiat Res* 153: 186–195.
- Mendez F, Sandigursky M, Kureekattil RP, Kenny MK, Franklin WA, Bases R. 2003b. Specific stimulation of human apurinic/apyrimidinic endonuclease by heat shock protein 70. *DNA Repair* 2: 259–271.
- Morimoto RI. 2002. Dynamic remodeling of transcription complexes by molecular chaperones. *Cell* 110: 281–284.
- Mosser DD, Caron AW, Bourget L, Denis-Larose C, Massie B. 1997. Role of the human heat shock protein hsp 70 in protection against stress-induced apoptosis. *Mol Cell Biol* 7: 5317–5327.
- Mosser DD, Caron AW, Bourget L, Merlin AB, Sherman MY, Morimoto RI, Massie B. 2000. The chaperone function of hsp70 is

- required for protection against stress-induced apoptosis. *Mol Cell Biol* 20: 7146–7159.
- Nadin SB, Vargas-Roig LM, Cuello-Carrion FD, Ciocca DR. 2003. Deoxyribonucleic acid damage induced by doxorubicin in peripheral blood mononuclear cells: possible roles for the stress response and the deoxyribonucleic acid repair process. *Cell Stress Chaperones* 8: 361–372.
- Nakamura J, La DK, Swenberg JA. 2000. 5'-Nicked apurinic/apyrimidinic sites are resistant to β -elimination by β -polymerase and are persistent in human cultured cells after oxidative stress. *J Biol Chem* 275: 5323–5328.
- Nakamura J, Swenberg JA. 1999. Endogenous apurinic/apyrimidinic sites in genomic DNA of mammalian tissues. *Cancer Res* 59: 2522–2526.
- Nakamura J, Walker VE, Upton PB, Chiang S-Y, Kow YW, Swenberg JA. 1998. Highly sensitive apurinic/apyrimidinic site assay can detect spontaneous and chemically induced depurination under physiological conditions. *Cancer Res* 58: 222–225.
- Nollen EAA, Morimoto RI. 2002. Chaperoning signaling pathways: molecular chaperones as stress-sensing 'heat-shock' proteins. *J Cell Sci* 115: 2809–2816.
- Novina CD, Sharp PA. 2004. The RNAi revolution. *Nature* 430: 161–164.
- Nylandsted J, Rohde M, Brand K, Bastholm L, Elling F, Jaattela M. 2000. Selective depletion of heat shock protein 70 (Hsp 70) activates a tumor-specific death program that is independent of caspases and bypasses Bcl-2. *Proc Natl Acad Sci U S A* 97: 7871–7876.
- Nylandsted J, Wick W, Hirt UA, Brand K, Rohde M, Leist M, Weller M, Jaattela M. 2002. Eradication of glioblastoma, and breast and colon carcinoma xenografts by Hsp 70 depletion. *Cancer Res* 62: 7139–7142.
- Polla BS, Kantengua S, Francois D, Salvioli S, Franceschi C, Marsac C, Cossarizza A. 1996. Mitochondria are selective targets for the protective effects of heat shock against oxidative injury. *Proc Natl Acad Sci U S A* 93: 6458–6463.
- Sadekova S, Lehnert S, Chow TY-K. 1997. Induction of PBP74/mortalin/Grp75, a member of the hsp70 family, by low doses of ionizing radiation: a possible role in induced radioresistance. *Int J Radiat Biol* 72: 653–660.
- Tang D, Xie Y, Zhao M, Stevenson MA, Calderwood SK. 2000. Repression of the HSP 70 promoter by NFIL6, Ku70, and MAPK involves three complementary mechanisms. *Biochem Biophys Res Comm* 280: 280–285.
- Vistica DT, Skehan P, Scudiero D, Monks A, Pittman A, Boyd MR. 1991. Tetrazolium-based assays for cellular viability: a critical examination of selected parameters affecting formazan production. *Cancer Res* 51: 2515–2520.