Heat shock protein 70 enhanced deoxyribonucleic acid base excision repair in human leukemic cells after ionizing radiation

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Abstract Base excision repair (BER) of DNA damage in irradiated THP1 human leukemic cells was stimulated by pretreating the cells with exogenous recombinant Hsp70. The treatment of THP1 cells with recombinant Hsp70 in cell culture promoted repair by reducing the frequency of apurinic, apyrimidinic (AP) sites in DNA before and after 1.3 Gy of radiation. However, by 30 minutes after 2.6 Gy, accelerated repair of abasic sites supervened, which may contribute to the loss of the very-low-dose cell hypersensitivity seen in clonogenic studies of other laboratories. After irradiation with 2.6 Gy, the crucial initial glycosylase step was markedly incomplete when cells had been transfected 24 hours before with a small interfering RNA (siRNA) designed to inhibit synthesis of Hsp70. In confirmation, lysates from irradiated siRNA-treated cells after 2.6 Gy were deficient in uracil glycosylase activity (UDG). Transfection with a scrambled RNA of the same size did not interfere with the glycosylase step, ie, the prompt conversion of damaged pyrimidine sites to abasic sites as well as the subsequent repair of those sites. BER measured by reduction of DNA AP sites before and after low-dose radiation was also deficient in THP1 cells that had been transfected with the siRNA designed to inhibit synthesis of Hsp70. These results implicate BER and the participation of Hsp70 in the repair of DNA in human leukemic cells with the doses of ionizing radiation used in clinical regimens.

INTRODUCTION

Stimulation of DNA base excision repair (BER) enzymes by their association with stress-inducible Hsp70B (Hsp70) was described previously (Mendez et al 2000, 2003a, 2003b; Kenny et al 2001). We reasoned that protection of cell viability after genotoxic agents would be correlated with the enhanced BER to be expected in THP1 human leukemic cells that had been pretreated with Hsp70, because THP1 cells take up exogenous Hsp70 in cell culture (Asea et al 2002). The viability of cells pretreated with exogenous Hsp70 before radiation was indeed protected. In contrast, transfection of the cells with a small interfering RNA (siRNA) designed to interfere with Hsp70 synthesis reduced cell viability after radiation (Bases 2005).

A variety of base damages is induced by radiation. The first repair step, which is performed by a DNA glycosy-

lase, removes damaged pyrimidine bases by hydrolysis of the base-sugar bond, leaving an abasic site. Failure to eliminate damaged bases could lead to cell cycle arrest or mutation. The resulting abasic site is essential in recruiting the cascade of repair activities; without the abasic site, repair is impossible. Abasic sites themselves are potentially lethal lesions which may lead to double-strand breaks unless they are promptly repaired (Kingma et al 1995). Low levels of abasic sites occur normally in cells by spontaneous base hydrolysis and free radical reactions. Damaged purines are removed by specific DNA glycosylases, but the resulting abasic sites are simultaneously removed by glycosylase-associated deoxyribose phosphodiesterase (dRPase) activity of those enzymes.

The enzyme activities in BER are as follows: glycosylase removal of a damaged DNA base, incision of sugar remnants at the 5' side by human AP endonuclease (HAP1) and excision at their 3' side and filling in the gap by DNA polymerase β ; all are stimulated in vitro by Hsp70. So far, the final ligase activity did not appear to

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Received 20 December 2005; Revised 1 May 2006; Accepted 19 May 2006.

be enhanced by Hsp70. Therefore, in living cells, the stimulation of DNA glycosylase activity would be expected to first enhance the appearance in DNA of apurinic, apyrimidinic (AP) sites. With progressive repair by HAP1 and DNA polymerase- β , AP sites then would be expected to diminish. In fact, time course studies compatible with this scheme were observed in experiments to be described.

Abasic sites (AP sites) in DNA of cells were determined using an aldehyde-reactive probe (ARP) reaction which is very sensitive and specific (Kuh0 et al 1992; Ide et al 1993; Nakamura et al 1998; Nakamura and Swenberg 1999; Atamna and Ames 2000; Mendez et al 2002).

We determined a potential protection mechanism by studying the ability of exogenous Hsp70 to enhance repair of DNA abasic sites after relatively low doses of radiation in THP1 cells. In accord with this notion, transfection of cells with siRNA designed to inhibit cellular synthesis of Hsp70 was found to interfere with glycosylase activity and BER. Results presented here and previously (Bases 2005) strongly implicate BER and its enhancement by Hsp70 as important factors in determining DNA repair and cell survival in the low dose shoulder region of radiation dose-response curves. These results are the first to correlate specific BER steps with the presence of Hsp70 in human cells in culture. In agreement with this, enhanced radiosensitivity and genomic instability in mouse cells deficient in Hsp70.1 and Hsp70.3 indicated the requirement for Hsp70 in normal DNA repair (Hunt et al 2004). Together, the results strongly implicate Hsp70 as an essential feature of repair of low-dose radiation damage in animal cells. Elimination of the very-low-dose (<1 Gy) hypersensitivity of cell viability to radiation by induced repair processes has been described by Marples and Joiner (1995). BER and the participation of Hsp70 may be features of the induced repair.

MATERIALS AND METHODS

THP1 leukemic cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells grew in RPMI 1640 medium with 10% fetal bovine serum, as previously described (Bases 2005).

Ionizing radiation

Cells were exposed to gamma radiation from a ^{137}Cs source at 7.64 Gy/min.

Isolation of cell DNA

Pelleted washed cells were lysed overnight at 4°C in 500 $\mu\text{g/mL}$ Proteinase K (Sigma, St Louis, MO, USA) in PBS with 0.02% sodium dodecyl sulfate (SDS). DNA was isolated by phenol/chloroform/isolym alcohol (25:24:1) ex-

traction. DNA was harvested by phenol extraction followed by elimination of RNA by digestion with T1 RNase (50 U/mL) and RNase A (100 $\mu\text{g/mL}$) in PBS for 30 minutes at 37°C.

ARP and slot blot assay

The procedures of Nakamura and Swenberg were followed with minor modifications. Briefly, AP sites in DNA were labeled with the biotin-containing ARP (Dojindo Laboratories, Gaithersburg, MD, USA). After binding to nitrocellulose for slot blots, streptavidin-labeled peroxidase was used to generate film exposure by chemiluminescence followed by quantitative densitometry (Mendez et al 2002).

Interfering RNA

Transfection with duplex siRNA designed to silence Hsp70 (Dharmacon M003501-03-05, Lafayette, CO, USA) was promoted by oligofectamine (Invitrogen, Carlsbad, CA, USA) (Bases 2005). Generally, 1–5 nm of interfering RNA was needed for 3×10^7 THP1 cells in a 10-mL suspension. Scrambled RNA of the same size was employed as a control. After 3 hours of transfection in serum-free RPMI 1640 at 37°C, cells were diluted to 5×10^5 cells/mL in complete growth medium for incubation overnight and their DNA was harvested, as described above.

Repair labeling of THP1 DNA with polymerase β

AP sites were first excised by digestion with HAP1, diluted to 0.08 pmol per 20 μg of DNA. HAP1 was a kind gift from Dr. Mark Kenny (Montefiore Medical Center/Albert Einstein College of Medicine, Bronx, NY, USA) who purified it from an expression plasmid provided by Dr. Ian Hickson (University of Oxford, Oxford, UK). After 30 minutes at 37°C, the digested DNA was purified by phenol extraction and precipitated from ethanol, rinsed, and dried (Kenny et al 2001). β -Polymerase synthesis to remove sugar remnants and fill in the single base gaps was done by incubating 2 μg of digested DNA with 1 U of β pol, 15 μCi of [α - ^{32}P]dGTP in 50 mM Tris-HCl (pH 8.0), 8 mM MgCl_2 , and 4 mM dithiothreitol (DTT) for 30 minutes at 37°C, followed by phenol extraction and ethanol precipitation (Mendez et al 2003). Small oligonucleotides and other labeled species were removed by filtration through a Sephadex G-25 column before counting the DNA in a scintillation counter by Cerenkov radiation.

Uracil-DNA glycosylase

DNA was digested by 30-minute incubation at 37°C with purified, truncated UDG Δ 84, a 27-kb recombinant en-

Fig 1. Inhibition of repair of AP sites in DNA of THP1 cells irradiated 1 day after transfection with siRNA to silence Hsp70. A total of 3×10^7 THP1 cells was transfected with 1.5 nm of of interfering RNA or with scrambled RNA for 3 hours employing oligofectamine. After incubation overnight in complete medium, 10^7 cells from each RNA treatment received 0, 1.3, or 2.6 Gy. DNA was isolated and assayed for abasic sites by the ARP method. Standard errors of the means in triplicate ARP assays are shown. The frequency of AP sites in DNA of cells that had been transfected with interfering RNA before receiving 2.6 Gy was significantly higher than with DNA from controls ($P \leq 0.02$). Untreated cells had 4.8 AP sites per 10^6 nucleotides; ▲, siRNA; ●, scrambled RNA.

zyme, a kind gift from Dr. Geir Slupphang (UNIGEN, University of Trondheim, Norway) in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid (HEPES) KOH (pH 7.8), 1 mM Na_2 ethylenediamine-tetraacetic acid (EDTA), and 5 mM DTT.

Western blot determination of THP1 cell lysate proteins

Twenty-four hours after transfection with 2.5 nm of siRNA or scrambled RNA, cells were given 0, 1.3, or 2.6 Gy. A total of 1.5×10^7 cells from each treatment group were sonicated in 1.5 mL of 0.1 N NaCl, 500 mM Tris (pH 7.5), 1 mM EDTA, 5 mM DTT, and 10 μM aprotinin (Sigma Aldrich, St. Louis, MO, USA). After 20 strokes of the sonicator on ice, only 1–2% of the cells appeared unbroken. Cell debris and intact cells were removed by centrifugation at $15\,500 \times g$ for 12 minutes, leaving 1 $\mu\text{g}/\mu\text{L}$

protein for analysis in a 7.5% acrylamide SDS gel and later electrotransferred to a nitrocellulose membrane.

The membrane first was blocked with 5% milk proteins; the first antibody was mouse monoclonal against Hsp70 (SPA-820, StressGen, Victoria, British Columbia, Canada); 10 μg was applied overnight in 20 mL of 5% milk proteins. After rinses, the second antibody, goat anti-mouse Ig precoupled with horseradish peroxidase (SAB-100, StressGen), was applied at 1/20 000 dilution for 2 hours before rinses and enhanced chemiluminescence (ECL).

Uracil-DNA glycosylase assay in cell lysates

Uracil-DNA glycosylase (UDG) activity in cell lysates was determined in a reaction measuring the release of ^3H -labeled uracil base. DNA substrate for this reaction was obtained by incorporation of [^3H]dUTP by nick translation (Sandigursky and Franklin 1999; Mendez et al 2000). The specific activity of the substrate was approximately 35 cpm/pmol. Released [^3H]uracil was recovered in supernatants from precipitated DNA substrates and were determined by scintillation counting.

RESULTS

siRNA reduced repair of AP sites

The frequency of AP sites in DNA of THP1 cells that had been transfected the day before with siRNA was found to be elevated before as well as after radiation. Radiation damage had apparently contributed to increasing the background frequency of AP sites, which are present as a feature of normal DNA repair. Although only 10 minutes at room temperature elapsed before isolating DNA from the irradiated cells, extra AP sites had been generated before they could be extinguished by endogenous repair enzymes (Fig 1). The level of unrepaired AP sites in DNA of cells after siRNA was 5.8 times greater than control values, consistent with partially disabled BER of preradiation AP sites. When both cell populations were irradiated, their DNA received equal burdens of new base damage, but the unrepaired AP site frequency after 1.3 Gy, or 2.6 Gy, remained 3.6 and 1.7 times greater in DNA of si-transfected cells, consistent with partially disabled repair by siRNA in those cells. To produce equivalent AP sites in cells after scrambled RNA transfection, 2.6 Gy were needed to equal the frequency obtained with 1.3 Gy in si-treated cells. After 1.3 Gy, the increase in AP sites over background levels in DNA of siRNA-transfected cells was twice as great as the increase found in control cell DNA.

Fig 2. Pretreatment of THP1 cells with Hsp70 left fewer AP sites after radiation. A total of 1×10^7 cells in 60 mL of complete growth medium were treated with 1.3 $\mu\text{g/mL}$ Hsp70, or growth medium alone for 4 hours. Cells were irradiated with doses shown and their DNA was isolated after incubation for 10 minutes at room temperature followed by 20 minutes at 37°C. O, No Hsp70; $\square$ Hsp70.

Exogenous Hsp70 promoted repair of AP sites after radiation

THP1 cells take up exogenous Hsp70 from culture medium as found by many laboratories. We showed that after 3- or 4-hour exposure to 1 $\mu\text{g/mL}$ in culture, the frequency of abasic sites in THP1 cell DNA was half of that in untreated cells (Bases 2005). Presumably, this was due to enhanced repair of the normal level of abasic sites. In a recent study on AP sites in DNA, after 4 hours of incubation with 1.5 $\mu\text{g/mL}$ Hsp70, slot-blot determination with ARP showed 343 ± 40 SE density units per 0.2 μg of DNA after Hsp70 exposure and 824 ± 20 U per 0.2 μg of DNA from untreated cells (not shown). The difference was significant at $P \leq 0.001$ by Student's test.

Results of the experiment of Figure 2 confirm the role of Hsp70 in enhancing BER. It showed that, after 1.3 Gy to Hsp70-treated cells, the level of DNA abasic sites was less than in untreated cells' DNA. However, after 2.6 Gy, the difference between the 2 treatment groups was not significant, presumably because of radiation induction of accelerated repair, thereby eliminating some features of

Fig 3. Time course of DNA repair after 2.6 Gy to THP1 cells. Transfection of 3×10^7 THP1 cells for 2.5 hours with either 2 nm of siRNA or 2 nm of scrambled RNA in 10 mL of transfection mix was followed by overnight incubation at 37°C in 66 mL of complete growth medium at 5×10^5 cells/mL. Two 10-mL portions of each of the 2 cell cultures were left unirradiated. Remaining cells received 2.6 Gy and were incubated at 37°C. At the times shown, 10-mL aliquots were removed for DNA isolation. Their DNA abasic sites were determined by the ARP assay and the [³²P]dGMP repair synthesis assay. Average values for the 2 unirradiated cell aliquots are shown. Open figures, ARP determination; solid figures, [³²P]dGMP incorporation. O, Scrambled RNA; $\square$, siRNA.

very-low-dose hypersensitivity (Marple and Joiner 1995). In the experiment of Figure 2, the cells in growth medium had been allowed to repair their DNA after 2.6 Gy during 10-minute incubation at room temperature followed by 20-minute incubation at 37°C before their DNA was isolated. In a separate experiment, repair at 37° was omitted but the time course was similar to that shown in Figure 2. The puzzling results after 2.6 Gy prompted the time course studies shown in Figures 3 and 4, which showed that the frequency of AP sites in irradiated cells followed a biphasic time course, unless they had received siRNA. Apparently, 2.6 Gy might have stimulated repair more than the exogenous Hsp70 employed in the study in Figure 2. This will be discussed.

AP sites after 2.6 Gy in si-transfected cells

In Figure 3, the frequency of AP sites in DNA of irradiated cells that had been transfected with scrambled RNA

Fig 4. A total of 6×10^7 THP1 cells was transfected with either 5 nm of siRNA or 5 nm of scrambled RNA. Cells were harvested 24 hours later and were given 2.6 Gy, followed by incubation at 37°C in complete medium, to accomplish repair. At the times shown, $\sim 10^7$ cells were removed for determination of abasic sites in their purified DNA by the ARP method. Lower panel, Slot-blot densities for 0.2- μ g DNA samples were determined in triplicate; standard errors of the means are shown. Values from 2 separate aliquots of unirradiated cells were determined in triplicate. Results of these 6 values were pooled and are shown at "0." ARP densities for DNA of cells after transfection with siRNA (Δ) or with scrambled RNA (O). At 30 minutes, the difference in AP site frequency for the 2 DNA's were significant at $P < 0.001$. Upper panel, Aliquots of the above DNA were digested at 37°C for 30 minutes with UDG to release damaged pyrimidines. For each 5- μ g DNA in 0.44 mL, UDG at a final concentration of 0.42 μ g/mL was used to remove susceptible bases. After phenol extraction and ethanol precipitation, the DNA was again reacted with ARP reagent. DNA samples (0.1 μ g) were determined in the slot-blot assay. After ECL treatment, films of the upper and lower panel studies were exposed for 30 and 20 seconds, respectively.

a day earlier first showed a decline after 2.6 Gy, followed by a rise and then progressive decline as repair proceeded during cell incubation for 60 minutes at 37°C. In contrast, DNA from cells pretreated with siRNA could only maintain their AP sites at a relatively constant level.

[³²P]GTP labeling of DNA at repair sites was more abundant in DNA from siRNA-treated cells, suggesting that more DNA was left unrepaired during postradiation incubation of the cells. Radiation-damaged DNA guanine bases that the endogenous cellular enzymes had failed to repair were then determined *in vitro*. After first removing AP sites from the purified DNA by *in vitro* treatment with HAP1, DNA polymerase β was used to insert α -³²P-labeled dGMP at the damaged sites. The lower portion of Figure 3 shows that the as-yet-unrepaired damage after 45 and 60 minutes of incubation of the cells was greater in DNA from siRNA-transfected cells than with DNA from cells that had been transfected with scrambled RNA. These results confirm the role of Hsp70 in stimulating repair of low-dose radiation damage in DNA in intact cells.

Time course determination of DNA abasic sites after 2.6 Gy

In repetition of the experiment of Figure 3, the lower panel of Figure 4 showed that, 24 hours after transfection of THP1 cells with the siRNA, the level of abasic sites was elevated. Presumably, endogenous HAP1 lacked the stimulation normally afforded by continued presence of Hsp70. In the experiment of Figure 4, the level of AP sites in DNA before irradiation of siRNA-treated cells was more than twice the level determined in cells that had received scrambled RNA ($P < 0.01$).

Promptly after 2.6 Gy the level of DNA abasic sites declined and remained low for at least an hour. By contrast, the level of AP sites in cells that had been transfected with scrambled RNA was initially lower, but then it rose to high levels before returning to baseline levels in a biphasic response. The lower panel of Figure 4 clearly delineates the biphasic response after 2.6 Gy for cells whose Hsp70 synthesis was left intact after transfection with scrambled RNA, in agreement with the results of Figure 3.

To learn whether progression of repair of radiation-induced DNA sites might correlate with the biphasic response shown in the lower panel of Figure 4, we reasoned that UDG-sensitive pyrimidine sites induced by 2.6 Gy would be present and that they could have been progressively removed by endogenous enzymes, leaving AP sites now detectable by the ARP reagent. In contrast, cells that received siRNA were deficient in generating AP sites by glycosylases. Those AP sites that were created were left as AP sites by partially disabled HAP1.

UDG is known to remove most radiation-damaged DNA pyrimidines (An et al 2005). Therefore, aliquots of the DNA of Figure 4 were subjected to UDG digestion *in vitro* before being reexposed to the ARP reagent. The upper panel of Figure 4 shows that, after 2.6 Gy or no irradiation at all, there was no difference in the AP site level in the DNA from siRNA-treated cells' DNA or scrambled RNA-treated cells' DNA. The biphasic response was abolished, because removal of damaged bases, which had not been achieved by glycosylases in the cells, was now completed by UDG digestion *in vitro*.

The absolute level of AP sites in the 2 panels of Figure 4 cannot be strictly compared because of slight differences in film exposure. Nevertheless, the level of AP sites detected in 0.1 µg of DNA after UDG and ARP reagent retreatment (upper panel) was approximately the same as found without UDG digestion in 0.2-µg aliquots (lower panel). This suggests that only half of the radiation-damaged DNA pyrimidines had been susceptible to cellular glycosylases when siRNA reduced the participation of Hsp701B.

Western blot studies of Figure 5A failed to show the "knockout" of Hsp70 synthesis in cells transfected with siRNA 24 hours previously. This might be accounted for by specificity of the siRNA used here. It is specific for Hsp701B and not Hsp701A or the 6 other members of the human heat shock protein 70s (Daugaard et al 2005). Furthermore, the turnover of Hsp701B may be slow enough to mask an siRNA-induced deficit. Zaarur et al (2006) noted that, despite depletion of HSF-1 in some human cells, background levels of Hsp72 were not altered. They suggested the intervention of alternative transcription factors. That may have been responsible for maintaining normal levels of Hsp70 in the results of Figure 5 with siRNA.

In fact, Figure 5B suggests that a minor Hsp70-related protein, 72 to 76 kDa of unknown significance, was indeed diminished in intensity in siRNA-treated cells. The results suggest the "knockout" consistent with the effect ascribed to siRNA.

siRNA-transfected THP1 cells after 2.6 Gy are deficient in UDG

One day after transfection with siRNA, THP1 cells exhibited 2 responses that differed from those of cells that had been transfected with scrambled RNA. First, after 2.6 Gy, the level of DNA abasic sites promptly and progressively declined from its previous elevated level to the lower one found in unirradiated cells that had been transfected with scrambled RNA (Figs 3, 4). Radiation stimulation of repair of abasic sites appeared to eventually override the previous inhibition of abasic site repair caused by siRNA

(Fig 1). In contrast, in irradiated control cells, the level of abasic sites rose and then declined.

This second feature in Figure 4 shows that after siRNA, the conversion of radiation-damaged bases to DNA abasic sites by glycosylase was markedly deficient. With the scrambled RNA-treated cells, radiation-damaged bases were rapidly converted to abasic sites in elevated levels that then declined; presumably, they were removed from DNA by the later steps of cellular BER, ie, HAP1 and polymerase-β. UDG activity in irradiated siRNA-treated cells appeared to have been deficient. Alternatively, supernormal levels of HAP1 could have very rapidly removed abasic sites.

To support the notion that UDG was deficient, UDG activity in lysates from irradiated siRNA- and scrambled RNA-transfected cells were compared. The lysates of the experiment of Figure 5 were studied. Results shown in Figure 6, A and B, clearly demonstrate deficient UDG activity in siRNA-treated cells after 2.6 Gy. These results provide a likely explanation for the failure of siRNA-treated irradiated cells to create the level of abasic sites found in DNA of the controls (Fig 4).

DISCUSSION

Results presented here are the first to correlate Hsp70 and specific BER step activity in irradiated human cells. Recent studies on DNA single- and double-strand break repair in mouse cells clearly implicated heat shock proteins in stimulating repair (Kabakov et al 2006). Their results support findings with human cells described in this report and previous studies from this laboratory.

After radiation, the frequency of potentially lethal DNA AP sites was elevated when cells had been pretreated with siRNA; it was reduced when cells were protected by pretreatment with Hsp70 (Figs 1, 2). The results are consistent with corresponding studies on cell viability (Bases 2005).

After 2.6 Gy, the level of DNA AP sites declined to a plateau in siRNA-treated cells. Presumably, radiation damage had stimulated BER without the intervention of Hsp70. But cells pretreated with scrambled RNA exhibited more abundant AP sites, in a biphasic response (Figs 3, 4). Twice as many AP sites were created, presumably by Hsp70 stimulation of pyrimidine glycosylases such as UDG, whose activity in siRNA-treated irradiated cell lysates was deficient (Fig 6).

Abasic sites in DNA are potentially lethal intermediates in the repair of the base damage inflicted by many agents, as well as abasic sites resulting from normal cellular processes. Detection of DNA abasic sites by the ARP procedure revealed that the removal of abasic sites in cells had been inhibited by earlier transfecting cells with the siRNA before low-dose radiation (Figs 1, 3, 4); abasic site

Fig 5. (A) The membrane of Figure 5A had been exposed to x-ray film for 10 minutes immediately after ECL development. Band densities in lanes 1,1, 2,2, 3,3 (siRNA-treated cell lysates) just above the main Hsp70 were apparently less than those in lanes 4,4, 5,5, or 6,6 (lysates from scrambled RNA-treated cells). Densitometer determination of these faint bands was too low for quantitation. Other extraneous families of bands are shown but no suggestion of si interference could be observed anywhere except as noted above for proteins in the 72–76 kDa mobility groups. (B) Western blot of proteins from THP1 cell lysates harvested 24 hours after transfection with siRNA (1–3) or scrambled RNA (4–6). Duplicate protein samples of 50 μ g each were placed on a 7.5% acrylamide gel. The 2 end lanes received 0.23 μ g of purified recombinant human Hsp70 (StressGen HSP-555). Gel electrophoresis continued at 225 V until the dye marker reached the gel bottom boundary. After transfer to a nitrocellulose membrane, bands were visualized by ECL staining after the 2 layered protocol described in Materials and Methods. Lysates from siRNA-treated cells that had received 0, 1.3, or 2.6 Gy are in duplicate lanes 1,1, 2,2, and 3,3. Lysates from scrambled RNA-treated cells are in duplicate lanes 4,4, 5,5, and 6,6. Lysates were prepared within 10 minutes after irradiation. The membrane was exposed to x-ray film for 5 minutes, 2 days after ECL to allow decay of signals from minor bands shown in Figure 5B. Densitometer values were Hsp70 (left): 526; Hsp70 (right): 611; Lysates; 1,1: 1018, 954; 2,2: 809, 769; 3,3: 904, 994; 4,4: 522, 606; 5,5: 773, 98; 6,6: 848, 838.

removal after 1.3 Gy was enhanced by pretreating cells with exogenous Hsp70 (Fig 2).

The time course studies of abasic sites in the DNA of THP1 cells that had received 2.6 Gy shown in Figures 3

and 4 might be explained as follows. During the 24 hours after transfection with the siRNA designed to inhibit elaboration of Hsp70, the activity of HAP1 in removing DNA abasic sites was expected to be depressed, because

Fig 6. Deficient UDg activity after 2.6 Gy in Hsp70 siRNA-transfected cells. (A) Left panel, Released [3 H]uracil from a DNA substrate by digestion with 20 μ g/mL lysates from unirradiated cells 24 hours after transfection with Hsp70 siRNA (●). Lysates from cells after 2.6 Gy (□). Lysates were from the experiment of Figure 5. Right panel, [3 H]uracil released by 20 μ g/mL lysates from cells that had been transfected with scrambled RNA. For comparison, the activity found after 30-minute digestion of the substrate with purified recombinant UDg Δ 84 (1.4 pg/ml) (⊗) is shown. With 100-fold higher concentrations of UDg Δ 84, 2×10^4 cpm were released, indicating complete release (not shown). (B) Repetition of the study of Figure 6A. The same substrates were used to test the lysates, but after loss of some activity after 4 cycles of freeze-thaw. Maximum expected [3 H]uracil release in A and B was 2×10^4 cpm. Symbols are as in Figure 6A. Results in another experiment, not shown, were virtually identical to that of Figure 6B. Lysates from cells that had received 1.3 Gy also were tested but are not shown. Results were intermediate between results with no radiation and 2.6 Gy.

Hsp70 is known to stimulate HAP1 activity (Kenny et al 2001). Accumulated AP sites were indeed 2.5 times more abundant than in DNA from cells that had been transfected with scrambled RNA (Fig 4). After 2.6 Gy, DNA damage in cells of both cultures was equivalent. Normal levels of Hsp70 in the cells that had earlier received only scrambled RNA was to be expected. In the latter culture, radiation-damaged pyrimidines would be removed at the normal rate by endogenous UDg.

In those control cultures, the frequency of AP sites indeed rose significantly by 30 minutes of repair incubation, and then it declined, perhaps normal processes having repaired the radiation-damaged pyrimidine sites by 70 minutes. In contrast, the frequency of abasic sites in the DNA of the siRNA-inhibited cells declined to a pla-

teau after radiation. Endogenous UDg in siRNA-treated cells were apparently deprived of Hsp70 stimulation. Both UDg and HAP1 probably proceeded to remove damaged bases and AP sites, albeit at reduced rates.

Treatment of the DNA with UDg in vitro abolished the difference in AP site frequencies (Fig 4, upper panel), unmasking DNA damage that might have been detected by ARP if Hsp70 had been expressed and functioned normally in enhancement of base excision enzyme repair. The contribution of damaged purines to AP site frequency may account for the persistent ARP reactivity after the treatment with UDg. Unfortunately, DNA glycosylases specific for damaged bases (eg, 8-oxoguanosine) such as OGG1 have associated drPase activity that abolish AP sites too (Sandigursky et al 1997; Rosenquist et al 2003).

So far, it has not been practical to perform studies parallel to those of Figure 4 for purine bases. The lower panel of Figure 3 does suggest siRNA induced deficiency in purine excision.

ARP values are equilibrium values. Therefore, they are the net result of DNA damage, glycosylase expression of AP sites, and HAP1 removal of AP sites. These equilibrium values for AP sites shown here and the enzyme-sensitive sites (ESS) potentially convertible to AP sites do not reflect the absolute frequency of all ESS induced by 2.6 Gy. Their sum, however, might be compared with previous estimates of ESS obtained from studies at the base sequence level of DNA from irradiated monkey CV1 cells.

ESS were determined in DNA at the base sequence level in previous studies of α DNA of irradiated monkey cells (Bases et al 1990). For sensitive sites at guanines (presumably also at adenines), 39×10^{-7} ESS/Gy were found; at thymidine (and also at cytosine) 6×10^{-7} ESS/Gy were found. Therefore, we estimate that, after 2.6 Gy, the sum was $10.1 + 10.1 + 1.6 + 1.6$ ESS per 10^6 nucleotides, or a total of 23.4 ESS per 10^6 nucleotides.

How does this estimate compare with the titer of abasic sites found by ARP determinations 30 minutes after 2.6 Gy, as determined in Figure 4? We estimate the initial ARP value as ~ 5 AP per 10^6 nt (Fig 1) (Bases 2005) with at least 3-fold increase by 30-minute incubation after 2.6 Gy; we then compare the AP site frequency of 5 ESS per 10^6 nt with the 23 ESS per 10^6 nucleotide estimate from DNA sequencing techniques above. These estimates are only approximations, subject to the broad assumptions noted. Nevertheless, the estimates from the 2 methods are within 10-fold of each other.

A mechanism of HSP elevation in tumor cells has been described, which involves stimulation of heat shock transcription factor 1, in an elaborate multistep scheme (Khaleque et al 2005). Regardless of the route that elevates HSP levels in tumor cells, the results described in this report clearly show that DNA BER is greatly influenced by Hsp70 and that it could be expected to influence cell survival after low-dose ionizing radiation, providing a possible mechanism for previous results on clonogenicity (Bases 2005). It is not possible to correlate all lethal events with refractory residual damaged pyrimidines or with abasic sites and the time course after ionizing radiation. The biphasic response of ARP reactivity (Figs 3, 4) makes this especially difficult. The biphasic response suggests repair of damaged DNA pyrimides. Nevertheless, the consequent release of AP sites entails the increased risk of their inducing cell-lethal double-strand breaks before they too are repaired.

Inhibition of BER in clinical radiation therapy may be practical. Low-dose radiation, 1.3 to 2.6 Gy, are typical of most clinical regimens. Tumor cell death induced by siRNA against Hsp70 using viral vectors has already

been found in certain model tumor systems (Nylandsted et al 2000, 2002). The present results suggest a target of opportunity for radiation oncologists. They should consider inhibiting the portion of DNA repair due to Hsp70 stimulation of repair enzymes to increase cell lethality in clinical radiation therapy regimens.

ACKNOWLEDGMENTS

The assistance of Frances Mendez and Margarita Sandigursky is greatly appreciated. Recombinant UDG Δ 84 was a kind gift from Dr. Geir Slupphang (Norwegian University of Science and Technology, Trondheim, Norway). Discussions with Dr. William Franklin are greatly appreciated. This work was supported by the Rome Sisters Foundation for Cancer Research.

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