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Hypothermia postpones DNA damage repair in irradiated cells and protects against cell killing

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Abstract

Hibernation is an established strategy used by some homeothermic organisms to survive cold environments. In true hibernation, the core body temperature of an animal may drop to below 0 °C and metabolic activity almost cease. The phenomenon of hibernation in humans is receiving renewed interest since several cases of victims exhibiting core body temperatures as low as 13.7 °C have been revived with minimal lasting deficits. In addition, local cooling during radiotherapy has resulted in normal tissue protection. The experiments described in this paper were prompted by the results of a very limited pilot study, which showed a suppressed DNA repair response of mouse lymphocytes collected from animals subjected to 7-Gy total body irradiation under hypothermic (13 °C) conditions, compared to normothermic controls. Here we report that human BJ-hTERT cells exhibited a pronounced radioprotective effect on clonogenic survival when cooled to 13 °C during and 12 h after irradiation. Mild hypothermia at 20 and 30 °C also resulted in some radioprotection. The neutral comet assay revealed an apparent lack on double strand break (DSB) rejoining at 13 °C. Extension of the mouse lymphocyte study to *ex vivo*-irradiated human lymphocytes confirmed lower levels of induced phosphorylated H2AX (γ -H2AX) and persistence of the lesions at hypothermia compared to the normal temperature. Parallel studies of radiation-induced oxidatively clustered DNA lesions (OCDLs) revealed partial repair at 13 °C compared to the rapid repair at 37 °C. For both γ -H2AX foci and OCDLs, the return of lymphocytes to 37 °C resulted in the resumption of normal repair kinetics. These results, as well as observations made by others and reviewed in this study, have implications for understanding the radiobiology and protective mechanisms underlying hypothermia and potential opportunities for exploitation in terms of protecting normal tissues against radiation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mrfmmm.2010.12.006 .

Conflict of interest statement

The authors declare that there is no conflict of interest.

Keywords

Hypothermia; Radioprotective effect; Cell survival; DNA damage

1. Introduction: why study hypothermia in radiation biology?

Exposure to ionizing radiation (IR) is unavoidable in many occupations associated with nuclear power generation, therapeutic and diagnostic radiology, and air travel. In addition, radiation therapy is a major cancer treatment modality resulting in increased patient survival [1]. However, unavoidable irradiation of bystander normal tissues during radiotherapy often results in a range of severe complications [2–4]. Radiation exposure is also one of the major problems facing future space travellers, potentially resulting in radiation sickness, cancer or death [5–7]. Therefore, efforts to reduce radiation toxicity is an active area of research. One major strategy includes the development of mitigators and radioprotectors [4,8]. However, a different approach emerges from several studies reporting that placing animals in hypothermic conditions where their core body temperature decreases well below 37 °C may provide effective means to protect against IR-induced damage [9–14].

The core body temperature has been considered a fundamental parameter in non-hibernating mammals which are maintained by numerous independent and redundant systems. However, that hypothermic approaches may be useful for radioprotection in humans appears more feasible with the documentation of several recent cases of accidental cold exposure which have shown that humans can survive in hypothermic conditions for considerable periods of time. Mitsutaka Uchikoshi survived unconscious for 24 days with core body temperatures of 22 °C. At –24 °C outside temperature, Erika Nordby was found alive with no heartbeat and a core body temperature of 16 °C [15]. When Anna Bågenholm was rescued from a lake trapped under a layer of ice after 80 min, she exhibited a core body temperature of 13.7 °C and lacked a heartbeat [16]. Although it took some time, all three victims have returned to almost completely normal states and Ms. Bågenholm has returned to her profession as a radiologist. In addition to these well-documented cases of accidental hypothermia, intentional hibernation for up to 7 months has been documented in a tropical primate [17]. Thus it appears that primates including humans may possess the potential to hibernate for extended periods of time.

In certain mammals such as the ground squirrel, *Citellus tridecemlineatus*, where hibernation is a default winter survival strategy, it has been reported that these animals exhibited increased survival after IR exposure when hibernating (5 °C) than when active (37 °C) [9]. Active animals exposed to 15 Gy total body irradiation (TBI) exhibited a mean survival time of 8 days while hibernating animals survived a mean of 66 days even if brought to active status immediately after irradiation. This is clearly a substantial difference at this dose associated with hematopoietic syndromes. The survival advantage decreased to a factor of two at 20 Gy, a dose associated with gastrointestinal syndromes and disappeared by 30 Gy [9]. This and other publications report dose reduction factors (DRFs) in the range of 1.5–3 for TBI doses up to 12 Gy [9–11,18–20]. Mice and rats, which do not hibernate but their body temperature can be lowered to ~28 °C with chlorpromazine, exhibited 73–100%

increased survival after TBI doses of 7–8 Gy under hypothermic conditions compared to normal [12–14]. Various protective physiological and biochemical effects have been reported in the tissues of hypothermic irradiated animals, including increased survival of intestinal crypt cells [11], less pronounced decrease in apoptosis and necrosis of blood lymphocytes, bone marrow hematopoietic cells, and thymus cells [21,22], more resistant proteinsynthesis in neurons, and less loss of cellular structural integrity [23,24].

Induction of reversible local hypothermia can result in improved outcomes for patients undergoing cancer radiotherapy, achieved by surface cooling, for example with ice packs applied to the patient's body, or circulation of cold air or water against the skin [25]. Placing ice pads around parasternal lymph nodes for 10 min before and 1 h after radiation treatment significantly limited skin desquamation in breast cancer patients, and local cooling with ice cubes during oral irradiation reduced patients' discomfort and prevented mouth mucosal inflammation [26]. Patients experienced less hair loss (alopecia) when scalp cooling was applied with palliative whole brain radiotherapy[27]. Reports of scalp hypothermia to prevent alopecia during chemotherapy have shown that the patients tolerate the cooling well and at least 50% of the patients have less or markedly less alopecia [3,28–30].

In contrast to local external hypothermia, induction of systemic hypothermia is much more challenging. Nevertheless, systemic induction of a hypometabolic state with profound hypothermia has been reported with infusion of cold saline or chemicals, for example, chlorpromazine hydrochloride which impairs temperature regulation [25,31]. In recent years there has been increasing recognition that even mild or moderate hypothermia may be a reasonable approach to treating a broad range of traumatic injury including brain trauma, cardiac arrest, or stroke as well as to provide protection against hypoxia and ischemia [25].

Various methods of inducing controlled hypothermia have been studied in mice. In one study, it was reported that mice breathing 80 ppm H₂S exhibited a 90% decrease in metabolic rate after 6 h and a decrease in core body temperature to 15 °C. When they were returned to a normal environment, no functional or behavioral differences were detected [32,33]. NASA Ames Research Center has a goal of developing means to place animals into prolonged states of minimal metabolism and to restore them to a normal state with no deficits. In one pilot study 6–8 week old C57BL/6J male mice with core body temperatures of 13 °C were exposed to 7 Gy TBI and allowed to recover to normal conditions. According to unpublished preliminary data, these mice survived the 30 day post-IR examination period. Thus being able to control the process of hypothermia may yield benefits in increased radioresistance and address NASA's interests in protecting astronauts from prolonged exposure to space radiation.

Mechanisms underlying the induction of hypothermia as well as its radioprotective effects are poorly understood. A lowered metabolic rate of tissues may lead to changes in the levels of oxygen-related metabolites due to decreased utilization of oxygen. In addition, proliferating cells may accumulate in more resistant phases of the cell cycle leading to alterations in the efficiency of DNA damage repair [11,31,34]. In cell culture, it has been reported that hypothermia increases cellular resistance to IR-induced DNA damage [35–37] including double-strand breaks (DSBs) [37], improves cell survival after IR and cytotoxic

drugs [31,34], and protects cells from cell death due to apoptosis [38]. The mechanisms through which hypothermia protects cells from apoptotic death induced by a variety of stress conditions have been shown to be both p53-dependent and p53-independent [38]. It also has been reported that hypothermia attenuates inflammatory responses by affecting multiple cytokines [18].

Here, we describe some initial studies on the survival of human cells in culture as well as the kinetics of induction and repair of DNA damage in hypothermic irradiated human BJ-hTERT cells and primary lymphocytes. We report here that hypothermia greatly reduces the rates of repair of DNA damage, and that the rates accelerate upon return to 37 °C. These results support the notion of “suspended animation” at the molecular level.

2. Materials and methods

2.1. Cells and temperature control

Human hTERT-immortalized BJ fibroblasts were a gift from Dr. Izumi Horikawa, NCI. The cells were cultured in 15% FBS–DMEM (Invitrogen). Whole blood lymphocytes were obtained from the NIH blood bank from paid healthy volunteers who gave written informed consent to participate in an IRB-approved study for the collection of blood samples for *in vitro* research use. The protocol is designed to protect subjects from research risks as defined in 45CFR46 and to abide by all internal NIH guidelines for human subjects research (protocol number 99-CC-0168). The lymphocytes were then separated from whole blood and cultured in 10% FBS–RPMI medium (Invitrogen, Carlsbad, CA).

All cultures were maintained at 37 °C, 5% CO₂, 20% O₂ according to recommended protocols. Three 1Q Thermo Scientific 120Q incubators (Fisher Scientific, Pittsburg, PA) were set up in a cold room to maintain the temperatures 13, 20, and 30 °C. The specific routines of cell cooling and warming for particular experiments are described in Section 3.

2.1.1. Immunocytochemistry—Human lymphocytes were plated into T-25 flasks which were sealed and kept at 37 °C or 13 °C for 2 h prior to IR. The lymphocytes were irradiated on ice with 0.6 or 2 Gy in a ¹³⁷Cs MARK I model irradiator (JL Shepherd & Associates, San Fernando, CA) with 2.4 Gy per min dose rate, or sham-irradiated lymphocytes were returned to the 37 °C or 13 °C incubators for various times (30 min–24 h). Two flasks for each dose were transferred from 13 °C to 37 °C at 8 h post-IR and incubated up to 24 h post-IR. The samples were further processed for fixation and immunostaining. The lymphocytes were fixed with 2% paraformaldehyde for 20 min at room temperature, washed twice with PBS and cytocentrifuged onto microscope slides. After 15-min rehydration in PBS, the slides were stored in chilled 70% ethanol and processed for immunofluorescent staining. The staining was performed as previously described [39], but PBS was replaced with PBS containing 0.5% Tween-20 and 0.1% Triton X-100 for blocking and antibody incubations. Primary mouse monoclonal anti-γ-H2AX antibody (Abcam, Cambridge, MA) and rabbit polyclonal anti-53BP1 (Novus Biologicals, Littleton, CO), and secondary goat anti-mouse Alexa-488 and rabbit Alexa-555-conjugated IgG (Molecular Probes, Eugene, OR) were used. Nuclei were either stained with PI, or unstained. Laser scanning confocal microscopy was performed with a Nikon PCM 2000

(Nikon, Inc., Augusta, GA). γ -H2AX foci were counted by eye, or their intensity was determined in ~100 cells per experimental point using the Adobe Photoshop software package (Adobe Systems, San Jose, CA). All data points represent averages for three donors.

2.1.2. OCDL analysis—Human lymphocytes were plated into T-25 flasks which were sealed and kept at 37 °C or 13 °C for 2 h prior to IR. Two Gy-irradiated or sham-irradiated lymphocytes were returned to the 37 °C or 13 °C incubators for various times (30 min–24 h). Two flasks for each dose were transferred from 13 °C to 37 °C at 8 h post-IR and incubated up to 24 h post-IR.

The High Pure PCR Template Kit (Roche, Indianapolis, IN) was used for isolation of DNA from lymphocytes as previously described [40,41]. To minimize oxidation artifacts during DNA isolation, all buffers were freshly prepared, autoclaved, purged with argon, and supplemented with 50 μ M phenyl-tert-butyl nitron, a free radical scavenger (PBN: Sigma–Aldrich, Saint Louis, MO) [42]. For OCDL detection and measurement, an adaptation of constant-field gel electrophoresis was used along with quantitative electronic imaging, and number average length analysis [43]. Human repair enzymes APE1 and OGG1 were used as damage probes along with *Escherichia coli* EndoIII (New England Biolabs, Beverly, MA) [41,42]. Detection of bistranded OCDLs was performed as previously described in [44]. DNA isolated from the lymphocytes was digested with human repair enzymes APE1 and OGG1, or *E. coli* EndoIII (New England Biolabs) as damage probes. OGG1 is expected to detect the majority of oxypurine clusters (containing at least one oxidized purine) and some abasic sites while EndoIII is expected to detect the majority of oxypyrimidine clusters (containing at least one oxidized pyrimidine) and a limited number of abasic sites. Using APE1 as a damage probe, we expected to detect the majority of abasic sites. For each enzyme-treated sample, a corresponding non-enzyme containing sample was also run as a control following the same steps but without the addition of enzyme. An adaptation of constant-field gel electrophoresis was used [43]. Electronic images for each gel lane were processed using QuantiScan (BioSoft, Ferguson, MO) and a densitograms were obtained. DNA standards (λ -Hind III Digest) were used to obtain the corresponding dispersion curve with Origin 6.1 (OriginLab, Northampton, MA). The numbers of average lengths (L_n) for each sample were calculated using the equations described in Sutherland et al. [43]. The frequencies of OCDLs were measured based on the L_n values of the enzyme-treated sample and the accompanying control sample.

2.1.3. Neutral comet assay—The single cell gel electrophoresis assay was performed using the CometAssay kit (Trevigen, Gaithersburg, MD). Briefly, BJ-hTERT fibroblasts were plated into 10-cm cell culture dishes on the day before an experiment. Then the dishes were sealed and transferred to the lower temperature incubators for 2 h prior to irradiation. The cells were processed for the comet assay directly after irradiation, or were either sham-irradiated or 2 and 10-Gy irradiated and allowed to incubate at their respective temperatures for 1, 4, and 24 h. Then, the cells were washed with cold PBS, scraped, collected into 1.5-ml tubes, and centrifuged. The cell pellet was then re-suspended in 10 volumes of low-melting agarose and 50 μ l was applied to the comet assay slide. The slides were dried completely at

4 °C for more than 30 min followed by incubation in Lysis solution at 4 °C for 30 min. After washing with TBE buffer, the samples were subjected to electrophoresis at 31V for 30 min in TBE and stained with SYBR Green. The signal was detected with Olympus fluorescent microscope (Olympus America Inc., Melville, NY). The tail length in more than 25 cells per slide was analyzed using IPLab imaging pathway software 4.0 (BD Biosciences, San Jose, CA).

2.1.4. Cell growth and survival—BJ-hTERT cells were plated at 37 °C into 10-cm cell culture dishes on the day before the experiment. The dishes were then sealed and transferred to the lower temperature incubators. The flasks were taken out of the incubators at 24 and 72 h, and cell growth was monitored with Zeiss optical microscope Axiovert 40C (Zeiss, Wellesley College, MA) and photographed with a Sony digital camera (Sony USA, New York, NY).

The colony formation assay in primary human fibroblasts has been described [45]. In our modified protocol, 250, 500, and 1000 cells were seeded at 37 °C into 10-cm cell culture dishes for 6 h to let the cells attach to the dishes, and 2 h prior to IR the dishes were sealed and transferred to the lower temperature incubators. The cells were irradiated on ice with 0.6, 2, 5, 7.5, and 10 Gy; control samples were sham-irradiated. Three dishes were used for each cell density and dose. The dishes were incubated at their respective temperatures for 12 additional hours, and then the cells were incubated at 37 °C for 8 days without replacement of the medium (see experimental scheme in Fig. 1C). The colonies were fixed with methanol, rinsed with PBS, stained with methylene blue (Sigma) for 2 h, and rinsed with water. Doubling time of BJ-hTERT fibroblasts is ~40 h, and after 8 days the cells form small discrete colonies which were counted using a magnifying glass (Fig. 1C). For each temperature setting the surviving fractions were normalized to the plating efficiency in sham-irradiated cells.

2.1.5. FACS analysis—BJ-hTERT cells grown under hypothermic conditions and at 37 °C were harvested by trypsinization and washed with PBS buffer. $1-2 \times 10^6$ cells in 100 µl PBS were fixed with chilled 70% ethanol by adding to cells dropwise and overnight incubation at 4 °C. The fixed cells were centrifuged and alcohol was removed. The cells were treated with RNase (100 units) (Sigma) for 20 min at 37 °C and stained with propidium iodide (PI) (25 µg) at 4 °C for 30 min, passed through 50 µm nylon mesh and acquired in FACS Calibure (BD Biosciences) by CELLQuest Pro software. The cell cycle analysis was performed using ModFit 3.0 software.

3. Results and discussion

3.1. Hypothermia studies in BJ-hTERT normal human fibroblasts

3.1.1. Survival of BJ-hTERT cells under hypothermic conditions—To help understand how hypothermia may protect cells from radiation exposure, we first determined to what extent various degrees of hypothermia affected the growth of immortalized normal human fibroblasts and to what extent the cells growing at different temperatures were protected from radiation exposure. BJ-hTERT fibroblast growth was inhibited at 13, 20, and 30 °C compared to 37 °C (Fig. 1A). At 13 and 20 °C, little if any change in cell position or

shape has taken place between 24 and 72 h after switching to the lower temperatures. FACS analysis of cell cycle distribution at low temperatures showed that hypothermia interfered with cell cycle progression (cells were in “dormant” state with low S-phase content but resumed normal cycling after transfer to 37 °C) (Fig. 1B).

The survival protocol involved plating unirradiated BJ-hTERT cells at 37 °C for 6 h, transferring the cultures to the desired hypothermic temperatures for 2 h before and 12 h after irradiation, then returning the cultures to 37 °C for 8 days, at which time clonal survival was measured (Fig. 1C). The unirradiated controls at the various temperatures revealed that hypothermia affected the plating efficiency (Fig. 1C, PE). This compromised survival may be evidenced by the increased number of “bright” cells under phase contrast, which may be apoptotic or otherwise incapable of attachment. HeLa carcinoma cells exhibited even lower PE under hypothermia than did BJ-hTERT cells and FACS analysis did reveal appreciable numbers of apoptotic cells (data not shown). The survival curves for BJ-hTERT cells (Fig. 1C, normalized to the corresponding PE) clearly demonstrate the radioprotective effect of hypothermia. The clonal survival of the cultures irradiated at 13 and 20 °C with doses up to 10 Gy appears to be independent of the dose at values between 80% and 100% of the control, while cultures irradiated at 37 °C exhibited decreasing clonal survival with increasing dose. For the 13 °C and 20 °C cultures, survival was improved by approximately 1.2-, 1.4-, and 2-fold, for IR doses of 5, 7.5, and 10 Gy, respectively. The degree of radioprotection for the 30 °C treatment was also apparently independent of dose up to 7.5 Gy but was lower at 10 Gy. The radioprotective effect of hypothermia may be connected to the dormant state of cells which indirectly supports the above-mentioned phenomenon of hypometabolic state under hypothermic conditions. It has been reported that colony-forming ability is maximal when cells are irradiated in the early post-mitotic (G0 or G1) and pre-mitotic (G2) phases of the cell cycle, and not in the mitotic (M) and late G1, or early S phases [46]. G1 arrest would allow time for IR-induced DNA damage repair prior to initiation of DNA synthesis, and prevent replication of a damaged template. Likewise, the G2 checkpoint is the last chance to block entry of damaged cells into mitosis [47]. Thus, the decrease of S phase cells in the hypothermic dormant state would potentially benefit cell survival. In addition, PE-normalized better colony formation ability could be explained by the fact that irradiated S phase cells are more likely to undergo apoptosis while more radioresistant G1 and G2 phase cells survive.

A previous report found 1.2-, 1.5-, and 1.7-fold improved survival for the tumor line MCF7 irradiated with 2, 3, and 4 Gy, respectively, at 2 °C [34], results consistent with our findings reported here. What is surprising in our findings is that a moderate lowering of temperature to 30 °C or 20 °C provides almost as much protection at a profound lowering to 13 °C. If this observation is applicable to whole animals and a lowering of core body temperature to between 20 and 30 °C provides a level of radioprotection similar to profound hypothermia, then the possibility of a feasible radioprotection protocol may be improved.

3.1.2. IR-induced DSBs in BJ-hTERT cells—To correlate DNA damage repair with incubation temperature, we subjected cells from this experiment to the neutral comet assay, which primarily measures DNA DSBs (Fig. 1D). The comet assay indicates that substantial numbers of DSBs are induced by 10 Gy at all four temperatures (13, 20, 30, and 37 °C),

however, there are considerable differences in the rates of DNA rejoining. While rapid DSB repair occurred at 37 and 30 °C, little if any repair occurred at 13 °C for at least 24 h post-IR. At 20 °C, while repair occurred more slowly than at the higher temperatures, the level of DSBs returned to near control levels by 24 h post-IR. The cells incubated at 13 °C contained as much DNA damage 24 h post-10 Gy as immediately after irradiation, but these cells also had greater survival at 10 Gy than those incubated at 30 or 37 °C, suggesting that unrepaired DSBs may not be detrimental to the cell at lower temperatures. However, the cells incubated at 20 °C exhibited the same degree of clonal survival as those at 13 °C, but in contrast to those cells, did exhibit DSB repair, albeit at slower rates than the cells at the higher temperatures. These results can suggest that DNA DSBs that are repaired slowly or not at all may not be detrimental to cell survival at greater IR doses and possibly may even be beneficial.

3.2. Hypothermia studies in human lymphocytes

3.2.1. Kinetics of γ -H2AX foci formation in human lymphocytes—Upon formation of a DSB, hundreds molecules of histone H2AX are phosphorylated in the chromatin flanking the break site [48]. The γ -H2AX focus assay is the most sensitive means to detect DSBs, but since the process requires the phosphorylation of H2AX and many other protein species, formation of the foci may be expected to be altered as the temperature decreases. It is known that cells irradiated on ice do not form visible foci until the temperature is returned to 37 °C [39], however, the complexity of focus formation makes it difficult to predict their kinetics at intermediate temperatures, such as 13 °C, a temperature commonly used in hypothermia and induced hibernation studies.

To gain some insight into how temperature affects γ -H2AX focus formation, we irradiated human lymphocytes *ex vivo* at temperatures of 37 or 13 °C (Fig. 2A). Lymphocytes were chosen for these studies as they are being used for monitoring IR responses in humans, macaques, and mice [48–50]. Comparison of the kinetics shows a slower rate of increase in the numbers of foci and intensity coupled with a lowered maximum at 13 °C compared to 37 °C. At 13 °C a 2.1- and 2.5-fold decrease in the maximal levels of γ -H2AX intensity was observed for both 0.6 Gy and 2 Gy IR, respectively, and a 2-fold decrease in numbers of foci was observed for 0.6 Gy. These numbers also agree with the approximately 2-fold reduction found in γ -H2AX intensity in lymphocytes from a single *in vivo* experiment with a mouse irradiated at NASA (Supplementary information) with a core body temperature of 13 °C compared to the 37 °C controls (data not presented). Indeed, that was the observation that prompted the whole study described in this paper.

In contrast to the rapid increase and decrease in the intensity of γ -H2AX foci in lymphocytes irradiated at 37 °C, at 13 °C their intensity did not decrease with time. Strikingly however, when at 8 h post-IR the lymphocytes were transferred from 13 to 37 °C, γ -H2AX intensity levels returned to background levels by 24 h post-IR (Fig. 2A). The lowered γ -H2AX levels after 0.6 and 2 Gy at 13 °C compared to 37 °C raise the question whether there were DSBs present without detectible foci at the lower temperature. However, since γ -H2AX formation at 13 °C is very slow, it is possible that some DSB repair took place, so that the focus numbers and DSB numbers would be concordant. On the other hand,

the results shown in Fig. 1D indicate that proper DSB repair does not occur at 13 °C in BJ-hTERT cells. If this were also the case in lymphocytes, then an increase in focus incidence might occur upon transfer of the cells to 37 °C. When this possibility was examined, a temporary increase was found to occur (Fig. 2A, middle panel), indicating that after 8 h at 13 °C, the cells still maintained a capacity for focus formation. This increase at 37 °C was followed by a decrease similar to that exhibited by the control cells at earlier times. Thus it appears that the lymphocytes at 13 °C did contain a number of DSBs lacking detectible foci after 8 h, and that the repair machinery was still capable of focus growth upon return to 37 °C.

3.2.2. DNA damage response (DDR) signal amplification at γ -H2AX foci—

Both the growth of H2AX foci at DNA DSB sites and the recruitment of DNA repair factors to those foci contribute to the signal amplification critical for efficient DNA damage repair. Two types of amplification were examined. First, the rates of focus enlargement at the two temperatures were analyzed. At 37 °C the foci enlarge rapidly reaching maximal size by 30 min post-IR, while no focus growth was observed at 13 °C (Fig. 2B, γ -H2AX columns and Fig. 2C). Thus, slow increase in focus numbers at 13 °C suggests initial H2AX phosphorylation at DNA damage sites without signal amplification.

Upon DNA DSB induction by IR, not only are γ -H2AX molecules formed at break sites, but many molecules of multiple DDR protein species also accumulate there. Thus staining for other DNA DSB repair proteins also results in visible foci [51,52]. However, γ -H2AX focus formation is necessary for proper DDR protein accumulation and rapid DSB repair, thus alterations in the kinetics of DDR factor recruitment to γ -H2AX foci should be expected.

Second, to determine how hypothermia affects DDR protein accumulation, we compared the initial kinetics of 53BP1 colocalization with γ -H2AX in human lymphocytes irradiated with 2 Gy at 13 and 37 °C (Fig. 2B). In contrast to the normal kinetics at 37 °C (Fig. 2B, left panel), at 13 °C detectible γ -H2AX foci are fewer in number, enlarge more slowly and fail to exhibit 53BP1 colocalization by 30 min post-IR (Fig. 2B, right panel). When after 8 h at 13 °C, the lymphocytes were returned to 37 °C, the γ -H2AX foci quickly enlarged to a size similar to that found under normal conditions and also exhibited 53BP1 colocalization (Fig. 2B, right panel, bottom row). These results suggest that DNA repair processes had become at least partially suspended at 13 °C, and had resumed upon return to 37 °C.

3.2.3. IR-induced oxidative clustered DNA lesions (OCDLs)—

IR induces multiple forms of DNA damage, some by direct interaction with the DNA bases and backbone, and some indirectly through ROS and NOS formation [40,53]. These lesions include single strand breaks (SSBs), oxidized bases and abasic sites, as well as DSBs [54–56]. Although among the most potentially lethal and oncogenic of DNA lesions, DSBs are a minor component among the types of IR-induced damage, being vastly outnumbered by the various oxidative lesions [56,57]. While single oxidative lesions are rapidly repairable, when one interferes with either DNA replication or transcription, a DSB may result [58–60]. A DSB may also result from the attempted simultaneous repair of two neighboring lesions [40,53,61].

In addition to the DSB, another potentially serious type of DNA damage occurs when two or more lesions are clustered within 10–20 base pairs of each other on opposite strands. The combination, named OCDL, may also be much more difficult to repair [54] due to the proximity of the multiple lesions. If OCDLs are left unrepaired or misrepaired, they may result in cell death and genome instability [62]. These lesions can be quantitated by digesting the modified DNA with enzymes that convert these lesions to single-strand breaks (SSBs), EndoIII at oxypyrimidine, OGG1 at oxypurine, and APE1 primarily at abasic sites. If two of these resulting SSBs occur within 10–20 base pairs of each other on opposite strands, a DSB forms, which can be assayed by constant field gel electrophoresis.

To examine the effect of hypothermia on OCDL repair, DNA was purified from lymphocytes taken from three normal donors, incubated at 13 or 37 °C, *ex vivo* irradiated with 2 Gy and analyzed for OCDLs (Fig. 3). The samples from the three patients exhibited similar behavior at the two temperatures. In all cases there was a rapid repair of the three types of OCDLs up to 2 h. At this time, there was a clear difference between the extent of repair of the samples at 13 and 37 °C, with approximately twice as many lesions remaining unrepaired at 13 compared to 37 °C. Interestingly, when samples that had been at 13 °C for 8 h were transferred to 37 °C, the rate of repair accelerated so that, with one exception, the levels of the various OCDLs decreased within an hour to the levels found in the samples that had been continuously incubating at 37 °C. This behavior is similar to that observed with γ -H2AX foci shown in Fig. 2A. After a transient rise in the incidence of the γ -H2AX foci immediately after the shift of the lymphocytes from 13 to 37 °C, the foci numbers dropped rapidly from the level in the continuous 13 °C samples to the level in the continuous 37 °C samples.

Both DNA DSBs and OCDLs are heterogeneous classes of lesions. Thus while it is possible that the lesions repaired early are the same as those repaired later, another possible explanation for the observed behavior is that some classes are more easily repaired than others and perhaps with different temperature effects. One might speculate that the plateaus reached after 2 h at 13 °C were due to the repair of a subset of OCDLs. The higher plateau level at 13 compared to 37 °C may be due to lesions the repair pathways for which contain a rate limiting step that requires a 37 °C environment. These lesions are apparently stable at 13 °C, because a shift to 37 °C results in their rapid repair. The rapid repair of OCDLs correlates with the transient peak of γ -H2AX foci, thus repair of some OCDLs may lead to DSB formation.

4. Conclusions

Studies of the potential uses of hypothermia for human benefit are just beginning. Accidental cases of profound hypothermia in humans have suggested that humans might be able to be placed in a state of “suspended animation” for lengthy periods of time. The ability to induce and control hypothermia may be useful for space travel. As humans travel longer and deeper into space, an increasingly serious issue facing future missions is prolonged radiation exposure. One solution may be “suspended animation”, with restoration of normal levels of activity, physiology, and neurological function when desired.

The challenge is to understand and control this process and many questions remain to be answered. Among the most obvious is how cold is cold enough to obtain a specific benefit. BJ-hTERT cells were protected against IR exposure at 30 °C as well as at 13 °C up to 7.5 Gy. Mice and rats exhibited increased radioresistance when their core body temperatures were lowered to 28 °C [12–14]. If sufficient, mild hypothermia would be easier to induce and make the process more feasible.

Another obvious question is whether after-the-fact hypothermia can be beneficial? In the case of some injuries involving tissue hypoxia, cooling the body mitigates the most serious outcomes. The protective mechanism involves minimizing reperfusion damage which arises from bursts of ROS induced by the sudden return of normoxia rather than modifying the original damage [25,63]. But accidental IR exposure does not involve hypoxia and DNA repair begins immediately. On the other hand, it takes days, weeks, and longer for the more serious results of radiation to manifest themselves—the hematopoietic, gastrointestinal, and neurological syndromes during which hypothermia may mitigate some of the worst outcomes. Thus it is an open question whether placing victims of accidental radiation exposure in hypothermic conditions would garner them any lasting benefit.

In the future, analysis of whole gene expression profiles under conditions of normal and hypometabolic states may help determine which genes are associated with stasis as well as with response to radiation exposure which would lead to a more complete mechanistic understanding of hypothermia effects and metabolic control. Already, some evidence indicates a functional significance of specific genes that are activated or inactivated in the hypothermic state [38,64].

Mitsutaka Uchikoshi recovered from being unconscious for 24 days with a core body temperature of 22 °C when found. This and other observations suggest that the notion of “suspended animation” may not be relegated to science fiction forever, but may at some time, after intense and thorough research become reality.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

BJ-hTERT cell growth and survival in hypothermic conditions. (A) BJ-hTERT cells remain attached and flattened in hypothermic conditions but grow more slowly compared to 37 °C. Representative images of the same fields at 24 and 72 h of incubation at 13, 20, 30, and 37 °C were obtained by phase contrast transmitted light microscopy. Magnification, 100×. (B) FACS analysis of cell cycle distribution in BJ-hTERT fibroblasts under normal and hypothermic conditions. Cells were plated at 37 °C for 6 h, grown at low temperatures for 12 h, and then transferred to 37 °C. Cell growth is inhibited under hypothermia but resumed when the cells are returned to 37 °C. The numbers are the S-phase fractions. (C) Clonogenic survival experiment at different temperatures. Experimental scheme and the plating efficiency (PE) at the different growth temperatures are shown. Graph shows clonogenic survival of BJ-hTERT cells after exposure to IR (0, 0.6, 2, 5, 7.5, and 10 Gy) and incubation at different temperatures (37, 30, 20, and 13 °C). Representative images of colonies in unirradiated and 7.5-Gy irradiated samples are shown (magnification, 200×). (D) Neutral comet assay for DSBs at 1 h before 10-Gy IR (white), and 0 (light gray), 1 (medium gray), 4 (dark gray) and 24 (black) h post-IR at the noted temperatures. Error bars denote 95% confidence intervals.

Fig. 2.

Analysis of γ -H2AX focus formation and 53bp1 accumulation in primary human lymphocytes. (A) γ -H2AX focus formation time course after lymphocyte IR exposure to 0.6 and 2 Gy. Intensity (top and bottom graphs) and the numbers (middle graph) of γ -H2AX foci were determined at 13 (filled circles) and 37 °C (open squares) for 24 h. At 8 h post-IR (black arrow), some samples were transferred from 13 to 37 °C (filled squares, dotted line) and incubated for further 16 h. Error bars are the standard deviations of 3 donor samples. (B) γ -H2AX focus formation (left columns) and 53BP1 accumulation at those foci (middle and right columns) at 37 (left panel) and 13 °C (right panel). Human lymphocytes were irradiated with 2 Gy and incubated for the noted times. Samples were fixed using 2% PFA and processed for γ -H2AX foci and 53BP1 analysis. Representative images show lymphocytes stained for γ -H2AX (green) and 53bp1 (red). Colocalized fluorochromes appear yellow in the merged images. The two proteins completely colocalized by 30 min post-IR at 37 °C, but did not colocalize at 13 °C until the cells are transferred to 37 °C. Magnification, 1000 $\times$. (C) γ -H2AX focus size was determined for least 100 cells per point in images from the experiment shown in the panels using Adobe Photoshop software. At 13 °C, γ -H2AX foci appear but remain small while at 37 °C, they continue to enlarge.

Fig. 3.

Analysis of oxidative clustered DNA lesions (OCDLs) in primary human lymphocytes.

Lymphocytes of three normal donors (A, red; B, blue; C, black symbols) were irradiated *ex vivo* with 2 Gy IR and incubated at either 37 (open squares) or 13 °C (open circles). Samples were taken at the noted times and the DNA digested to determine the number of (left) oxypyrimidine (EndoIII) (middle) oxypurine (OGG1), and (right) abasic sites (APE1) per Gbp. Some samples were transferred from 13 to 37 °C after 8 h (filled diamonds, dashed line). The dot-dashed line represents the value of unirradiated control samples. Data plotted individually for each donor.