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Survival Responses of Human Embryonic Stem Cells to DNA Damage

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Abstract

Pluripotent human embryonic stem (hES) cells require mechanisms to maintain genomic integrity in response to DNA damage that could compromise competency for lineage-commitment, development and tissue-renewal. The mechanisms that protect the genome in rapidly proliferating hES cells are minimally understood. Human ES cells have an abbreviated cell cycle with a very brief G1 period suggesting that mechanisms mediating responsiveness to DNA damage may deviate from those in somatic cells. Here, we investigated how hES cells react to DNA damage induced by ionizing radiation (IR) and whether genomic insult evokes DNA repair mechanisms and/or cell death. We assessed response parameters and markers that monitor induction of pathways controlling hES cell survival, including Caspase-3 and -8, Ki67, as well as components of the ATM/p53/p21 pathway. We compared the expression of the anti-apoptotic protein survivin in hES cells, normal diploid somatic WI38 cells and MDA-MB-231 cancer cells. We find that hES cells respond to DNA damage by rapidly inducing phospho-H2AX foci, phosphorylation of p53 on Ser15 and p21 mRNA levels, as well as concomitant cell cycle arrest in G2 based on Ki67 staining and FACS analysis. Unlike normal somatic cells, hES cells and cancer cells robustly express the pro-survival protein Survivin, consistent with the immortal growth phenotype. SiRNA depletion of Survivin diminishes hES survival post-irradiation. Thus, our findings provide insight into pathways and processes that are activated in human embryonic stem cells upon DNA insult to support development and tissue regeneration.

Keywords

Survivin; p53; ATM; cell cycle; DNA damage; embryonic stem cells

Introduction

Human embryonic stem (hES) cells are pluripotent progenitors that can produce the three embryonic germ layers and support post-natal tissue-renewal. Thus, it is important for these cells to have mechanisms to protect genomic integrity and avoid proliferative defects that may debilitate development or cause lethality. Human ES cells exhibit an abbreviated cell cycle due to a brief G1 period [Becker et al., 2006]. These cells lack a traditional Restriction point in late G1 (reviewed in [Blagosklonny and Pardee, 2002]), but maintain stringent control of histone gene expression at the G1/S phase transition [Becker et al., 2007]. Regulatory mechanisms for transcriptional control at the onset of S phase are operative [Becker et al., 2007; Ghule et al., 2007]. The regulatory machinery for histone gene expression is spatially organized in discrete foci immediately following completion of mitosis [Ghule et al., 2007], and this organization is distinct from that observed in normal somatic cells [Ghule et al., 2008] or tumor cells (Ghule et al, 2009 or unpublished results??). Human ES cells exhibit a robust induction of components of the DNA damage response [Becker et al., 2007], but mechanisms that mediate cell survival have not been examined.

The pathways controlled by the ataxia telangiectasia-mutated (ATM) and ATM-related (ATR) proteins represent the principal pathways by which cells react to DNA damage in somatic cells. ATM is activated upon γ -ionizing radiation (IR)-induced DNA double strand breaks, phosphorylates p53 on serine 15, followed by upregulation of p21 mRNA levels in somatic human cells (reviewed in [Gartel and Radhakrishnan, 2005]). This induction of p21 has also been observed in hES cells [Becker et al., 2007], suggesting that hES cells are capable of blocking CDK levels to ensure a cell cycle arrest. A key question that remains to be addressed is how hES cells regulate cell survival following DNA damage and whether the cell cycle is inhibited. Although DNA damage response mechanisms have been examined in mouse ES cells [Aladjem et al., 1998; Lavin and Kozlov, 2007; Schmidt-Kastner et al., 1998; Hong and Stambrook, 2004; Hirao et al., 2000], mouse and human cells differ in their ability to be immortalized and thus survival mechanisms may be fundamentally distinct.

In somatic cells, mechanisms are operative that enable cells to stop cell cycle progression and to make molecular decisions to repair DNA and promote cell survival, or to undergo apoptosis to ensure genomic integrity within the organism (reviewed in [Altieri, 2008b; Altieri, 2008a; Salz et al., 2005; Luo and Altieri, 2008]). ES cells exhibit an immortalized phenotype that resembles the growth phenotype of cancer cells, which prevent apoptosis through induction of the anti-apoptotic protein survivin, while normal somatic cells have minimal survivin expression [Altieri, 2006; Altieri, 2003]. In this study, we have characterized the cell survival response of human ES cells following IR-induced DNA damage, in relation to expression of survivin. Using a combination of biochemical and cellular approaches, we show that DNA-damaged human ES cells have decreased cell survival, are able to block in the G2 phase of the cell cycle, have a functionally activated ATM pathway, and express survivin.

Materials and Methods

Cell lines and Irradiation

The H1 hES line (WA01, WiCell Research Institute, Madison, WI; <http://www.wicell.org/>) was used in this study. The control cell lines WI-38 (normal somatic lung fibroblast) and MDA-MB-231 (metastatic breast cancer) were used for comparison. A ^{137}Cs source irradiator was used to apply 5 Gy of IR to the cells.

H1 Cell Culture Conditions

Human embryonic stem (hES) cells were cultured under non-differentiating conditions in hES cell culture medium (80% DMEM/F12, 20% KnockOut-Serum Replacement, 2 mM L-glutamine, 1% non-essential amino acids (NEAA), 0.1 mM 2-mercaptoethanol (all from Invitrogen, Carlsbad, CA), 4ng/mL basic fibroblast growth factor (R+D Systems, City/State)) at 37°C, 5% CO₂ and high humidity. Irradiated mouse embryonic fibroblasts (iMEF), isolated from day 13.5 embryos of CF-1 mice (Charles River Laboratories, City/State), were used as feeder cells for the H1 culture. iMEFs were cultured until passage 3 in DMEM (Hyclone, City/State) supplemented with 10% heat inactivated fetal bovine serum (Hyclone) and 1% NEAA (Invitrogen). Cells were then mitotically inactivated by irradiation at 5000 rad (Cesium 137) before seeding on a 0.1% Type A gelatinized (Sigma) 6-well plate at 1.75×10^6 cells/plate.

At approximately 80% confluence (day 6) the H1 culture was collected using Collagenase Type IV (Invitrogen) at a concentration of 1 mg/mL. This solution (1 mL) was added to each well of a 6-well plate and incubated at 37°C and 5% CO₂ for 5 min. When the edges of the hES cell colonies began to detach, the plate was removed from the incubator and transferred to a sterile biological safety cabinet. The collagenase was removed and 2 mL fresh hES cell culture medium was added to each well. A 5 mL glass pipette was used to further dislodge the H1 colonies from the plate and the contents of each well were collected and transferred to a sterile 50 mL conical tube. Fresh hES cell culture medium (3 mL) was used to rinse any remaining cells from the wells and was added to the same tube. The cells were centrifuged at 1000 rpm and 23°C for 5 min, after which the supernatant was removed and the pellet was resuspended in 24 mL fresh hES cell culture medium.

After removing the iMEF culture medium from new feeder cells and rinsing with 2 mL/well Dulbecco's Phosphate Buffered Saline without calcium and magnesium chloride (Invitrogen), each well was preloaded with 1.5 mL fresh hES cell culture medium, followed by the addition of 1 mL of the H1 suspension previously collected. This 1:4 seeding ratio provided two 6-well plates at approximately 15% confluence 24 h later. At this time, the exhausted hES cell culture medium was removed and replaced with 2.5 mL/well fresh medium. This procedure was repeated at 48 and 72 h, concluding with the H1 cell irradiation 2 h after the final feeding (day 3).

Survival and Apoptotic Activity

Adherent H1 cells were collected at 0 h without IR and at 7 h both with and without IR and stained with Trypan Blue to determine H1 survival post-IR. Caspase-3 and 8 activities were then measured via colorimetric enzyme activation assays (Chemicon/Millipore, Billerica, MA) at 0 h without IR and at 2, 3, and 4 h time points with IR to examine activation of apoptotic pathways according to the manufacturer specifications.

Flow Cytometry

H1 cells were collected at 0, 2, and 7-h, with IR at 2 and 7 h and without IR at 0 and 7 h and processed for cell cycle flow cytometry to examine cell cycle distribution. Cells were trypsinized, washed with phosphate-buffered saline, fixed in 95% ethanol overnight at 4°C, stained with propidium iodide and analyzed using FACSCalibur (Becton, Dickinson Biosciences, San Jose, CA) and ModFit software (Verity Software House, Topsham, ME).

Protein Analysis

Protein and immunohistochemistry samples were collected at 2, 7, 14 and 24 h time points after IR, with unirradiated controls also being collected at 0, 7 and 24 h. Protein was used to analyze by western blot analysis the ATM pathway-related proteins γ H2A.X (Upstate/

Millipore, Billerica, MA), p53 (Santa Cruz, Santa Cruz, CA), p53 ser15 (Cell Signaling, Danvers, MA), p21 (Santa Cruz), and Chk2 ser68 (Cell Signaling), as well as survivin (Novus Biologicals, Littleton, CO). Alpha tubulin (Santa Cruz) was used as an internal control. Immunohistochemistry was used to analyze γ H2A.X, p53 ser-16, and Ki67 (Abcam, Cambridge, MA). Ki67 yields staining patterns specific to different phases of the cell cycle and was used to determine whether H1 cells block in cell cycle in response to IR to support flow cytometry data.

Reverse transcription-quantitative PCR (RT-qPCR)

mRNA levels were analyzed as described previously described [Becker et al., 2007]. Briefly, total mRNA was extracted from hES cells using TRIzol reagent according to the manufacturer specifications (Invitrogen) and subjected to DNase I digestion, followed by column purification using the DNA Free RNA Kit (Zymo Research, Orange, CA). Reverse transcription was performed using iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Quantification was performed on ABI PRISM 7000 sequence detection system with SYBR Green supermix (Applied Biosystems, Foster City, CA). Human-specific p21 sequence was used as previously reported [Becker et al., 2007]. Mitochondrial cytochrome c oxidase (m-COX) was used as an internal control (reverse: 5'-CGG GAA TTG CAT CTG TTT TT-3', forward: 5'-GGC CAC CAA TGG TAC TGA AC-3').

Cell Proliferation Assay

An MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt] assay was used to analyze cell viability. For each time point, H1 cells in a 6-well plate were washed with PBS twice. The number of surviving cells was assessed by the determination of A490 nm of the dissolved formazan product after addition of MTS for 1 h, as described by the manufacturer (Promega, City/State).

siRNA Transfection

Twenty-four hours before transfection, 70-80% confluent H1 cells were split 1:5 and plated on top of MEF cells in a 6-well plate. Cells were transfected with oligofectamin (Invitrogen) according to the manufacture's instruction. The final concentration of control or survivin siRNA is 50 nM. The medium was replaced 6 h later, and the cells were analyzed for survivin expression 48 h after transfection.

Results

Survival of human ES cells is significantly decreased by ionizing radiation

To address cell survival of human ES cells in response to DNA damage, we initially determined the number of viable adherent H1 human ES cells following γ radiation (S Grags??). IR treatment of human ES cells decreases cell viability by approximately 65% at 7 h after treatment (Fig. 1A). Populations of adherent cells at 2, 3 and 4 h were subjected to molecular analyses of pathways activated in response to IR. The induction of apoptotic mechanisms was monitored by assessing the activities of Caspase-3 and Caspase-8 (Fig. 1B). While both Caspase-3 and -8 are induced by ~2 fold at 2 h post-radiation, Caspase-3 levels remain modestly elevated at 3 and 4 h, while Caspase-8 levels continue to increase to ~9-10 fold over control levels by 4 h. Thus, at 4 h after DNA damage, mechanisms supporting programmed cell death have been initiated that account for the observed loss of hES cell survival at 7 h (Fig. 1A).

Human ES cells arrest in G2 upon γ -irradiation

To determine whether human ES cells are capable of blocking cell cycle progression after IR, we used a combination of Ki67 nuclear staining and cell cycle analysis by flow cytometry. Ki67 immunofluorescence staining gives distinct nuclear patterns for different stages of the human ES cell cycle [Solovei et al., 2005; Becker et al., 2006; Ghule et al., 2007]. Using Ki67, we find that H1 human ES cell populations are deficient for both G1 and M phase cells after irradiation (Fig. 2A). For example, mitotic cells represent ~5% of the actively proliferating population that did not receive radiation but are undetectable at 2 h after irradiation, indicating that irradiation prevents new mitotic divisions. Mitotic cells gradually increase during the subsequent time-points (7, 14 and 24 h) as cells recover from DNA damage and resume mitosis (Fig. 2A). Consistent with an irradiation induced cell cycle block by 2 h, we find that the distribution of cells in late G1 and S versus G2 phase is significantly altered (Fig. 2A). The disappearance of G1 and M phase cells, along with increased representation of cells in G2 (at 2 hr), suggests a block in the G2 phase of the cell cycle.

Given the abbreviated G1 period in human ES cells and the Ki67 nuclear staining patterns we observed, the possibility arises that these pluripotent cells may not undergo a G1 block upon IR and may move into S phase or undergo cell death. To examine this possibility, we performed cell cycle analysis by flow cytometry. At 2 h post irradiation there is evidence of significant cell death as indicated by the accumulation of a sub-G1 cell population (Fig. 2B). The data reveal an increase of G2 cells after IR, with 14% before irradiation increasing to 24% by 7 h after irradiation. These data are consistent with the findings obtained with Ki67 (see Fig. 2A) and indicate that DNA-damaged hES cells can undergo apoptosis and/or arrest in G2.

The ATM pathway is activated in human ES cells in response to IR

To determine if the ATM pathway is functionally activated in human ES cells in response to IR, we analyzed several major components of the ATM pathway. Immunofluorescence microscopy reveals that irradiated human ES cells have an increased number of foci containing ATM-phosphorylated H2A.X (γ H2A.X foci), which reflect double-stranded DNA breaks (Fig. 3A), as well as an increase of p53 phosphorylated on serine 15 (Fig. 3B). This activation of p53 occurs within 2 h after IR and results in approximately 15-fold upregulation of p21 mRNA levels (Fig. 3C). Western blot analysis of ATM pathway-related proteins corroborates the immunofluorescence data (Fig. 3D). For example, γ H2A.X, p53-phosphoSer15, and Chk2-phosphoSer68 are all increased by 2 h after IR. Although phosphorylation of p53 and Chk2 has subsided by 7 h, phosphorylation of H2A.X is sustained until at least 24 h. This transient induction of phospho-Chk2 hES cells is consistent with the observed G2/M block at 2 h and its resolution by 7 h after IR based on Ki67 staining (see Fig. 2A). Interestingly, p21 protein levels in hES cells are barely elevated post-irradiation (Fig. 3D) despite a robust induction at the mRNA level (see Fig. 3C). Thus, H1 human ES cells exhibit an activated ATM pathway after IR treatment.

The DNA damage response of human ES cells differs from responses in somatic cells

The DNA damage response has been well characterized in both normal somatic and tumor-derived human cells. Therefore we compared activation of the ATM pathway in H1 embryonic stem cells with normal diploid WI-38 fibroblasts and MDA-MB-231 breast cancer cells (Fig. 3A and 3D). Notably, γ H2A.X protein levels in both H1 and MDA-MB-231 cells decrease more slowly than in normal somatic WI-38 cells, indicating possible delay in or decreased ability to repair double-stranded DNA breaks. In contrast, p53 phosphorylation peaks by 2 h and decreases by 7 h after IR in both H1 and WI-38 cells, while elevated p53 phosphorylation is maintained for at least 24 h in MDA-MB-231 cells,

perhaps due in part to high basal levels of phospho-p53. Chk2-Ser-68 phosphorylation is transiently induced at 2 h in both H1 and MDA-MB-231 cells but not detectable in WI-38 cells under our experimental conditions. Protein levels of p21 are barely detectable in irradiated H1 cells, while p21 is clearly detected and induced by p53 activation as expected in WI-38 cells; p21 is not expressed in the tumor-derived MDA-MB-231 cells. Taken together, these results indicated both differences and similarities in the molecular mechanisms by which human ES cells mitigate deleterious effects of DNA damage compared to normal and tumor-derived somatic cells.

Expression and siRNA-mediated depletion of survivin during the DNA damage response in human ES cells

Both control and irradiated H1 human embryonic stem cells have substantial levels of survivin, comparable to levels in MDA-MB-231 cancer cells, as determined by western blot analysis (Fig. 4A). These high levels of survivin that are indicative of resistance to apoptosis are largely sustained for at least 24 h in both cell types, but survivin is not detected in WI-38 fibroblasts irrespective of irradiation. These data suggest that although H1 cells do undergo cell death in response to IR, survivin may contribute to cell survival in irradiated human ES cells.

To test whether survivin is required for hES cell survival after induction of DNA damage, we generated survivin-depleted ES cells using RNA interference. Survivin siRNA treatment results in a >3-fold decrease in survivin protein levels in non-irradiated cells (Figs. 4B and 4C). This reduction in survivin levels does not impinge on survival during the first 24 h after IR (Fig. 4B), but appears to reduce cell fitness modestly at later times (96 h) (Fig. 4C). We conclude that survivin is at least in part dispensable for hES cell survival after gamma radiation, suggesting a novel role in human ES related regulatory mechanisms.

Discussion

In this study we have shown that human ES cells lack a G1 checkpoint in response to ionizing radiation, but appear to block in the G2 phase of the cell cycle. We find that the ATM pathway is activated in hES cells in response to IR, but there is essentially no p21 to support a cell cycle arrest. However, these cells activate Chk2 and express survivin, thus providing p21-independent mechanisms to recuperate from DNA-damage.

In response to DNA damage human ES cells will not divide but instead initiate apoptosis. Indeed, we find that the majority of hES cells are undergoing cell death via Caspase-related mitochondrial apoptosis following induction of DNA damage. This apoptotic mechanism may ensure that genomic integrity is not compromised in human ES cells. Our results show that surviving human ES cells enter a cell cycle block after IR. Human ES cells do not arrest in G1, but block in the G2 phase of the cell cycle.

Survivin is expressed in hES cells, but its depletion does not have a major impact on hES cell survival after DNA damage. This finding suggests that survivin may perform another function that may not necessarily be coupled to canonical cell survival pathways. Survivin function is associated with inhibition of mitochondrially-mediated and Caspase-related programmed cell death [Altieri, 2008b; Altieri, 2008a; Salz et al., 2005; Luo and Altieri, 2008; Altieri, 2006; Altieri, 2003]. Recent studies have shown that Caspases are transiently expressed in human ES cells during the transition when ES cells relinquish pluripotency and initiate lineage commitment [Abdul-Ghani and Megeney, 2008; Janzen et al., 2008; Fujita et al., 2008]. This increased Caspase activity is required to degrade the complement of transcription factors that establish and/or sustain the pluripotent state. The possibility arises

that survivin may attenuate the activity of Caspase to permit cell survival while allowing the degradation of the regulatory factors that determine stemness.

In this study we have revealed some of the previously unknown fundamental differences between human ES cells and normal somatic cells in response to IR. We have shown that human ES cells lack a G1 checkpoint and have a p21-independent G2 checkpoint. Further elucidation of the mechanisms governing the G2 checkpoint in human ES cells will give us insight into how these cells are protecting their genomic integrity. In addition, we conclude that human ES cells possess an inherent novel role of survivin. Dissecting the role of survivin in human ES cells may provide revelations in the process of undergoing cellular differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Decreased cell survival of human ES cells upon γ -irradiation. A. Survival of H1 cells was determined by assessing the number of adherent live cells (as determined by trypan blue staining) per well of a 6-well plate before and at 7 h after IR with 5 Gy. Values represent the average of three determinations within a representative experiment and the error bars are based on the standard error. B. Induction of apoptosis was determined by monitoring the activities of Caspase-3 and Caspase-8 by enzymatic assays at 2, 3 and 4 h after IR. Values represent the average of sample duplicates. Standard error was determined, but this error is minimal and not readily visible in the graph).

Fig. 2.

γ -Irradiation induces a G2 phase related cell cycle arrest in human ES cells. A. Immunofluorescence microscopy was used to examine Ki67 staining patterns (n=200; bar graph in upper portion), which reflect different stages of the cell cycle (lower portion), as a time-course after γ -irradiation (5 Gy). Values represent the average of three independent determinations of 200 cells per sample within a representative experiment and the error bars are based on the standard error. B. Fluorescence activated cell sorting was used to assess the distribution of hES cells in different cell cycle stages following γ -irradiation. We note that the fraction of cells in G1 may be over-estimated due to the presence of inactivated mouse embryonic fibroblasts that are arrested in G1.

Fig. 3. Activation of the ATM pathway activation by IR in hES cells. A. Immuno-fluorescence microscopy was used to examine the in situ appearance of γ H2A.X foci, which reflect phosphorylation of H2A.X by ATM at sites of double-stranded DNA breaks after IR. B. Same as A, but assessing phosphorylation of p53 at Ser-15 by in situ immunofluorescence microscopy after IR. C. Quantitative real-time PCR analysis was used to show that in situ activation of the ATM pathway after IR results in the expected p53 mediated induction of p21 mRNA levels at 2 and 7 h after radiation with 5 Gy. Error bars represent standard deviation of sample duplicates. D. Western blot analysis was used to show activation of components of the ATM pathway in human H1 embryonic stem cells, normal diploid WI-38 fibroblasts and MDA-MB-231 breast cancer cells upon γ -irradiation. Blots were prepared with antibodies probing ATM dependent phosphorylation of H2A.X (γ H2A.X) and Ser-15 phosphorylation of p53, as well as the levels of the CDK inhibitor p21 and Chk2, using α -tubulin as an internal control for protein loading.

Fig. 4.

Expression and function of survivin upon γ -irradiation in human ES cells. A. Western blot analysis was used to monitor survivin protein levels in human H1 embryonic stem cells, normal diploid WI-38 fibroblasts and MDA-MB-231 breast cancer cells upon γ -irradiation (5 Gy) at the indicated time-points. Levels of α -tubulin were determined as an internal control for protein loading. B. Effect of siRNA-mediated depletion of survivin during the DNA damage response in human ES cells. Cells were transfected with either control or survivin siRNA for 48 hours and then exposed to 5Gy of γ -irradiation for 7 min. siRNA treatment is effective as revealed by reduced survivin levels throughout a 96 h time course after IR (lower portions of Panels B and C). Cell survival was measured by MTS assay as function of time after treatment (graphs in upper portions of Panels B and C).