Mutation of the Hematopoietic Cell Phosphatase (Hcph) Gene Is Associated with Resistance to \( \gamma \)-Irradiation-Induced Apoptosis in Src Homology Protein Tyrosine Phosphatase (SHP)-1-Deficient "Motheaten" Mutant Mice

Hui-Chen Hsu, Leonard D. Shultz, Xiao Su, Jian Shi, Ping-Ar Yang, Melissa J. Relyea, Huang-Ge Zhang and John D. Mountz

*J Immunol* 2001; 166:772-780;

doi: 10.4049/jimmunol.166.2.772

http://www.jimmunol.org/content/166/2/772

---

**Why The JI?**

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

---

**References**

This article cites 47 articles, 24 of which you can access for free at:

http://www.jimmunol.org/content/166/2/772.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Mutation of the Hematopoietic Cell Phosphatase (Hcph) Gene Is Associated with Resistance to γ-Irradiation-Induced Apoptosis in Src Homology Protein Tyrosine Phosphatase (SHP)-1-Deficient “Motheaten” Mutant Mice

Hui-Chen Hsu,* Leonard D. Shultz, † Xiao Su,‡ Jian Shi,§ Ping-Ar Yang,* Melissa J. Relyea,† Huang-Ge Zhang,* and John D. Mountz2,*§

To determine the role of Src homology protein tyrosine phosphatase (SHP-1) in the ionizing radiation-induced stress response, we analyzed the apoptotic response and cell cycle function in irradiated spleen cells of motheaten (me/me) mice. The defect in me/me mice has been attributed to mutations of the Hcph gene, which encodes SHP-1. Homozygotes develop severe systemic autoimmune and inflammatory disease, whereas heterozygotes live longer and develop hematopoietic and lymphoid malignancy. Spleen cells from C57BL/6 (B6)-me/me and B6-/+ controls were analyzed after γ-irradiation from a 137Cs source. B6-me/me cells were significantly more resistant than B6-/+ cells to γ-irradiation-induced apoptosis exhibiting a higher LD_{50}. The defective apoptosis response of the B6-me/me cells was exhibited by T and B cells and macrophages. Of the Bcl-2 family members analyzed, a significantly more resistant than B6-/+ cells, but not B6-me/me cells. Analysis of 3,3′-dihexyloxacarbocyanine iodide revealed resistance to the γ-irradiation-induced mitochondrial transmembrane permeability transition in the B6-me/me cells. The blocking of the cell cycle in the G_{2}/G_{1} phase characteristic of the irradiated B6-/+ cells was not observed in the B6-me/me cells. There was decreased phosphorylation of p38 mitogen-activated protein kinase and increased phosphorylation of p53 from spleen cell lysates of irradiated B6-me/me mice compared with wild-type mice. These data suggest that SHP-1 plays an important role in regulation of apoptosis and cell cycle arrest after a γ-irradiation-induced stress response. The Journal of Immunology, 2001, 166: 772–780.
induction of the mitochondrial membrane permeability transition collapse that leads to the activation of downstream caspase(s) and the execution of Fas-triggered apoptosis.

Considering the importance of tyrosyl phosphorylation and dephosphorylation as the second messenger in the γ-irradiation-induced genotoxic stress response, we hypothesized that SHP-1 is an important signaling molecule for γ-irradiation-induced apoptosis in mice. This hypothesis was tested by irradiating spleen cells obtained from homozygous C57BL/6 (B6)-me/me mice and wt B6-+/+ mice with a moderate dose of 137Cs. There was a markedly greater resistance to γ-irradiation-induced apoptosis in the spleen cells of B6-me/me mice, which was accompanied by a failure in the arrest of the irradiated cells at the G1/G2 phase. This was associated with defects in up-regulation of Fas transfection, defects in mitochondrial transmembrane potential changes, a decrease in phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK), and an increase in phosphorylation of p53. Our results suggest that after a genotoxic stress, the HcpH gene product, SHP-1, is critically involved in signaling the induction of apoptosis and cell cycle arrest in response to γ-irradiation in mice.

Materials and Methods

Mice

Heterozygous B6-HcpHmeme/+ breeding pairs were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were kept in a room equipped with an air filtering system. The cages, bedding, water, and food were sterilized, and the mice were handled with sterile gloves. All mice used in the present experiment were homozygous B6-me/me mice between 2 and 3 wk old. Age-matched wt B6-+/+ mice were used as controls.

Cell lines

Stable By55.6 double-positive hybridoma (BYDP) cell lines expressing either wt or mutant SHP-1 (SHP-1 ΔP) were a gift from Dr. Ulrike Lorenz (University of Virginia, Charlottesville, VA) (16). Briefly, BYDP hybridoma cells were cotransfected with the expression vector pEBG alone or pEBG constructs, into which wt or mutant SHP-1 containing a deletion in its phosphatase domain (aa 451–475) (SHP-1 ΔP) had been inserted, together with the plasmid pMHiNeo, which confers G418 resistance. Clones were selected for G418 resistance, and expression of SHP-1 has been confirmed by immunoprecipitation followed by immunoblotting (16).

Cell preparation and γ-irradiation

All mice were sacrificed at 2-3 wk of age. Spleens were removed and single-cell suspensions were prepared with RBC being removed by lysis in buffer containing 0.15 M NH4Cl, 1.0 mM KHCO3, and 0.1 mM sodium EDTA. After washing twice with RPMI 1640 medium, the remaining cells were resuspended in culture medium and kept on ice. The cells were either unirradiated (0 Gy) or irradiated with γ-rays from a 137Cs source (Gamma cell 100; Nordion, Ontario, Canada) at a dose of 5, 15, or 30 Gy. After irradiation, the cells were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at a concentration of 5 × 106 cells/ml medium in a flat-bottom six-well plate at 37°C in a 5% CO2 chamber until the indicated time point of analysis. All reagents used for cell culture were obtained from Life Technologies (Grand Island, NY).

Analysis of cell viability

Cell viability was determined using the 7-amoactinomycin D (7-AAD) method (see below) and is indicated as the percentage of viable cells compared with the viable unirradiated control cells.

Analysis of apoptotic cells

Apoptosis induced by γ-irradiation was determined by flow cytometry using 7-AAD staining (17). Briefly, cells were harvested at the indicated time point after irradiation. They were stained with 20 μg/ml of 7-AAD (Calbiochem, La Jolla, CA) in FACS buffer (5% FCS and 0.1% sodium azide in PBS) on ice for 30 min in the dark. After washing twice with FACS buffer, the cells were fixed in 1% paraformaldehyde/FACS solution supplemented with 10 μg/ml of actinomycin D (Calbiochem) and analyzed using a FACSscan (Becton Dickinson, Mountain View, CA). Analysis was conducted using 10,000 cells per sample. The histogram analysis was performed using WinMDI Software (obtainable at Trotter@scripps.edu). Three different populations were defined according to 7-AAD vs cell size. The 7-AADlow cell population represents the nonapoptotic cells, the 7-AADmid cell population represents the apoptotic cells, and the 7-AADhigh population that exhibits low light scatter represents cell debris. To determine the dose of γ-irradiation required to reduce the cell viability by 50% (LD50 values), the logarithm of γ-ray dose (0, 5, 15, 30 Gy) was plotted against the number of viable cells as the percentage of unirradiated controls. In some experiments, spleen cells were stained with fluorochrome-conjugated anti-CD3 (clone 145-2C11) (PharMingen, San Diego, CA), anti-B220 (clone RA3-6B2) (PharMingen), anti-CD11b (Mac-1, α-chain, clone M1/70; PharMingen), or anti-CD5 (clone 53-7.3; PharMingen) before staining with 7-AAD to determine the phenotype of the apoptotic cells. Briefly, cells (1 × 10⁶) were incubated first with unconjugated anti-CD16/CD32 (Fc Block; PharMingen) to block the background staining with PE-conjugated Ab. Cells were next stained with the fluorochrome-conjugated Ab at room temperature for 20 min, then washed once with FACS buffer before 7-AAD staining as described above.

Measurement of mitochondrial transmembrane potential dissipation

The dissipation of the mitochondrial transmembrane potential was evaluated by the loss of the uptake of the fluorescent probe 3,3′-dihexyloxacarbocyanine iodide (DiOC6) (18). A single-cell suspension (1 × 10⁶ cells/ml) was stained with 2 μM DiOC6 and Hoechst-33342 dye (50 ng/ml) at 37°C for 15 min. The stained cells were resuspended in FACS buffer for FACS analysis to quantitate the disruption of mitochondrial membrane potential according to the reduction of cells stained positive with DiOC6. Some stained cells were washed once with PBS, resuspended in a minimum amount of PBS, and analyzed microscopically (Nikon, Melville, NY) using a DMS510 filter (Nikon) to visualize DiOC6 staining of living cells and a UV-1A filter (Nikon) to visualize Hoechst-33342 staining of dead cells.

RNA isolation

Total RNA was extracted using the RNA-STAT 60 Reagent according to the manufacturer’s protocol (Tel-Test, Friendswood, TX). In brief, 10 × 10⁶ spleen cells were harvested from cell culture plates, lysed in 1 ml RNA-STAT 60 reagent, extracted with 0.2 ml of chloroform, and centrifuged at 12,000 × g for 15 min at 4°C. The aqueous phase was transferred to a fresh tube. RNA was precipitated with 0.5 ml of isopropanol and centrifuged at 12,000 × g for 15 min at 4°C. RNA pellets were washed with 1 ml of 75% ethanol, centrifuged at 7500 × g for 5 min at 4°C, and air dried.

Examination of gene expression using an RNase protection assay

A multiprobe RNase protection assay was performed using a RiboQuant MultiProbe RNase protection assay kit according to the manufacturer’s protocol (PharMingen). Briefly, on day 1, the mouse APO-2 and APO-3 RNA template sets were labeled with [γ-32P]UTP (3000 Ci/mmol; Amer sham, Arlington Heights, IL) at 37°C for 1 h. The reaction was terminated by incubating the reaction mixture with DNase. The labeled probe was then purified using phenol/chloroform/isomyl alcohol extraction followed by precipitation with 4 M ammonium acetate and ice-cold ethanol at −70°C for 30 min, pelleted by spinning in a microcentrifuge at 4°C for 15 min, washed once with ice-cold 90% ethanol, air dried, and resuspended in the hybridization buffer. Hybridization was conducted by incubating the probe with 5% of the RNA sample at 56°C overnight in a 90°C prewarmed heat block. The next day, the hybridized RNA samples were digested with both RNase and proteinase K to digest the unprotected RNA. The samples were further extracted, precipitated, and washed as described above for day 1. They were resuspended in 1× loading buffer and then denatured at 90°C for 3 min before loading on to a 5% acrylamide gel. The electrophoresis was conducted by incubating a 10% acrylamide gel with 30 μl of the RNA sample set at 30 V for 2 h. After the electrophoresis, the gel was exposed onto a filter paper using a Bio-Rad Gel Dryer (model 583; Bio-Rad) at 80°C for 1.5 h. The dried gel was exposed to an x-ray film (Kodak X-AR; Kodak, Rochester, NY) at −70°C for 2 days. The radioactivity was quantitated by scanning the dried gel using phosphoimaging (Molecular Dynamics, Sunnyvale, CA) and analyzed with ImageQuant and PhosphorImager software (Molecular Dynamics). The housekeeping gene, GAPDH, was used as the internal control. The radiointensity of each gene was standardized with the intensity of GAPDH.
Cell cycle analysis using FACS analysis of propidium iodide (PI)-stained cells

Cell cycle kinetics was analyzed by flow cytometry analysis of cells stained with PI (19). In brief, cells (2 x 10⁶) were harvested from the culture plates at the indicated time points. The cells were washed twice with PBS and fixed in 70% ice-cold ethanol at 4°C on ice for at least 30 min. Residual RNA in the cells was removed by incubating the cells with RNase (50 μg/ml; Sigma) at 37°C for 30 min at a constant shaking rate (150 rpm). Cell pellets were then resuspended in a solution containing 50 μg/ml of PI in PBS and stored in the dark at 4°C until the FACS analysis was performed. The PI-DNA complex was analyzed on a Coulter EPICS PROFILE II flow cytometer using an argon ion laser with a constant wavelength of 488 nm. Cell debris and doublets were excluded. Twenty-five thousand cells were counted for each analysis. Cell cycle compartments, including sub G0, G0/G1, S, and G2/M phases, and the percentage of cells in the compartments was analyzed using EPICS Cytologic software. The histogram analysis was performed using Modfit LT Software (Verity Software House, Topsham, ME).

Western blot analysis

Unirradiated or irradiated spleen cells from B6-/+ or B6-me/me mice or stable SHP-1 wt or stable SHP-1 ΔP BYDP cells were cultured and harvested at different time points. Cell pellets were washed once with PBS and lysed by SDS sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 50 mM DTT, and 0.1% (w/v) bromophenol blue. The cell lysates were sonicated for 2 s using a 550 Sonic Dismembrator (Fisher Scientific, Suwanee, GA) to shear DNA. The samples were then heated at 95°C and loaded on to a 12% polyacrylamide gel. Each lane was loaded with the lysate from an equivalent number of cells. After electrophoresis, the gels were blotted onto nitrocellulose membranes, which were then incubated with blocking buffer containing 1% TBS, 0.1% Tween 20, and 5% (w/v) nonfat dry milk for 1 h at room temperature. Phosphorylation of proteins was detected using an anti-phospho-p44/42 MAPK (early response kinase (Erk)1/2) (Thr202/Tyr204), an anti-phospho-p38 MAPK (Thr180/Tyr182), an anti-phospho-JNK/SAPK (Thr183/Tyr185), and an anti-phospho-p53 (Ser15) (New England Biolabs, Beverly, MA). The primary Ab was detected with an HRP-conjugated anti-rabbit IgG secondary Ab and visualized by chemiluminescent detection (Kirkgaard & Perry Laboratories, Gaithersburg, MD).

Statistical analysis

The results are expressed as mean ± SEM. The two-tailed Student’s t test was used for statistical analysis when two different groups of samples were compared. A p value of <0.05 was considered statistically significant.

Results

Greater resistance of spleen cells of B6-me/m to γ-irradiation-induced cell death

Spleen cells isolated from B6-me/m mice and age-matched wt B6-/+ mice were irradiated with various doses of γ-irradiation including 0, 5, 15, and 30 Gy, from a 137Cs source. The percentage of cells surviving 6 h after the irradiation was plotted against that of the unirradiated control (Fig. 1). The percentages of B6-me/m spleen cells that survived after 5 and 15 Gy of irradiation were significantly higher than the percentages of control B6-/+ cells that survived at this level of irradiation. The percentage of B6-me/m cells that survived 5 Gy was 74 ± 4%, whereas 53 ± 3% of B6-/+ cells survived (p < 0.01, spleens of three mice in each group). After 15 Gy, 67 ± 2% of B6-me/m spleen cells and 35 ± 6% of B6-/+ spleen cells survived (p < 0.01, n = 3). After 30 Gy, the survival rate for both groups fell to <40% (39 ± 1 and 31 ± 8% for B6-me/m vs B6-/+ mice, respectively; p > 0.05). The doses of γ-irradiation required to induce a 50% decrease in the viability (LD50) of B6-me/m spleen cells and control B6-/+ spleen cells 6 h after γ-irradiation were calculated. The dose of γ-irradiation required to induce a 50% decrease in the number of viable cells of B6-me/m mice was 24.5 Gy, whereas for B6-/+ mice was only 6.5 Gy. This result suggests that the spleen cells of SHP-1 mutant B6-me/m mice exhibit a markedly increased resistance to γ-irradiation-induced cell death.

Lower levels of apoptosis of B6-me/m spleen cells upon exposure to ionizing radiation

The induction of apoptosis after irradiation of the spleen cells derived from B6-me/m mice and control B6-/+ mice with 137Cs was measured by quantitating the percentages of 7-AAD<sup>+</sup> cells detected by flow cytometry (Fig. 2A). The percentages of cells undergoing spontaneous apoptosis 6 h after γ-irradiation were similar in both groups (8 ± 5 vs 11 ± 7% in B6-me/m vs control B6-/+ spleen cells. p > 0.05). The percentage of the apoptotic cells 6 h after treatment of B6-me/m spleen cells with 137Cs (5 Gy) was 21 ± 5% (Fig. 2A, bottom left), which was significantly lower than the percentage of apoptotic cells in the irradiated B6-/+ spleen cell population (40 ± 4%, Fig. 2A, bottom right). The sensitivity of B6-me/m cells and control B6-/+ cells to 137Cs treatment at 5 Gy was compared at various time points for up to 24 h after irradiation (Fig. 2B). The maximal difference in sensitivity was observed 12 h after irradiation with the percentage of apoptotic cells in B6-me/m mice at this time point being 33 ± 3% and that of B6-/+ mice being 71 ± 5% (p < 0.005). These results suggest that SHP-1 functions as an important regulator of the induction of apoptosis during the γ-irradiation-induced genotoxic stress response.

Resistance to γ-irradiation-induced cell death in all major populations of immune cells from B6-me/m mice

The predominant cell types in B6-me/m and wt mice differ, with lymphocytes predominating in the spleens of wt mice, whereas the spleen cells of B6-me/m mice are enriched in macrophages and granulocytes (20–22). Moreover, the population of CDS<sup>+</sup> B cells is larger in the spleens of B6-me/m mice (23, 24). We used flow cytometry to determine whether there were differences in the susceptibility to apoptosis of T lymphocytes, B lymphocytes, CD11b<sup>+</sup>, and CD3<sup>+</sup> cells 6 h after γ-irradiation (5 Gy). The significantly decreased apoptosis and significantly higher survival rate of spleen cells from B6-me/m mice compared with B6-/+ mice after γ-irradiation was observed in all the cell types analyzed (Fig. 3). Among these, the T cells and B cells from B6-me/m mice showed the greatest difference in sensitivity to γ-irradiation-induced cell death compared with the equivalent cell types from wt mice. There was significantly increased resistance to γ-irradiation-induced apoptosis in cells from irradiated B6-me/m mice compared with B6-/+ mice and a correspondingly increased survival rate of the different populations of T cells, B cells, and macrophages from irradiated spleen cells from B6-me/m mice compared with B6-/+ mice (see Table I).

Spleen cells from B6-me/m mice exhibited defective γ-irradiation-induced mitochondrial membrane potential disruption

The disruption of the mitochondrial transmembrane potential has been reported to be one of the main factors leading to induction of apoptosis in response to γ-irradiation (25, 26). Spleen cells from either B6-/+ or B6-me/m mice were either unirradiated (0 Gy) or irradiated with 5 Gy and cultured for an additional 6 or 24 h (Fig. 4). After 6-h culture, most unirradiated cells showed maintenance of the mitochondrial potential with DiOC<sub>6</sub> uptake (green staining) and low nuclear staining indicated by Hoechst-33342 (blue staining) (A and B). At 6 h after 5 Gy-irradiation was observed in all the cell types analyzed (Fig. 3). Among these, the T cells and B cells from B6-me/m mice showed the greatest difference in sensitivity to γ-irradiation-induced cell death compared with the equivalent cell types from wt mice. There was significantly increased resistance to γ-irradiation-induced apoptosis in cells from irradiated B6-me/m mice compared with B6-/+ mice and a correspondingly increased survival rate of the different populations of T cells, B cells, and macrophages from irradiated spleen cells from B6-me/m mice compared with B6-/+ mice (see Table I).

FIGURE 1. Decreased sensitivity to γ-irradiation-induced cell death in B6-me/m spleen cells. Single-cell suspensions prepared from B6-me/m or control B6-/+ mice were either unirradiated (0 Gy) or γ-irradiated using a 137Cs source at a dose of 5, 15, or 30 Gy. After 6 h, the numbers of viable cells were determined by FACS analysis using 7-AAD staining (7-AAD<sup>+</sup> large cell population) and are indicated as the percentage of the unirradiated control (**, p < 0.01). Cells from at least three mice were used in each group.

Downloaded from http://www.jimmunol.org/ by guest on November 7, 2017
γ-irradiation, there was an increase in the percentage of cells with low DiOC6 staining and an increase in nuclear staining in cells from wt mice compared with B6-<sup>me/me</sup> mice (C and D). At 24 h after γ-irradiation, the majority of cells from wt mice lost uptake of DiOC6, whereas cells from spleen cells of B6-<sup>me/me</sup> mice retained positive staining for DiOC6 dye and negative staining for the Hoechst 33342 dye (E and F).

To further quantitate the defect in membrane mitochondrial permeability transition in B6-<sup>me/me</sup> mice after irradiation, single-cell suspensions from B6-<sup>me/me</sup> and B6-/+ mice were irradiated with 5 Gy, stained as described above, and analyzed by flow cytometry analysis. γ-irradiation-induced mitochondrial permeability transition was indicated by a decrease in DiOC6 fluorescent intensity of irradiated cells (solid curves) compared with unirradiated cells (open curves) (Fig. 5A). In B6-/+ mice, 3 h after irradiation, 78% of unirradiated cells exhibited high DiOC6 staining compared with 75% in irradiated cells (Fig. 5A, top left). At 3 h, there was a significant decrease in the cells from B6-<sup>me/me</sup> mice were DiOC6<sup>+</sup> (69%) and there was no difference in unirradiated compared with irradiated cells.

This suggests that at early time points, there is a spontaneous decrease in DiOC6 staining in unirradiated cells from B6-<sup>me/me</sup> mice compared with irradiated cells from B6-/+ mice (Fig. 5A, top). Six hours after γ-irradiation, most of the irradiated (60%) and unirradiated (62%) spleen cells from B6-<sup>me/me</sup> mice exhibited high staining of DiOC6 (Fig. 5A, middle left). In contrast, there was a marked decrease in DiOC6 staining in irradiated cells from B6-/+ mice (32%) compared with unirradiated cells (61%) at 6 h. At 24 h after γ-irradiation, most of the spleen cells from B6-<sup>me/me</sup> mice exhibited high DiOC6 staining after irradiation (66%) compared with unirradiated (76%) cells. By 24 h after irradiation of cells from B6-/+ mice, most irradiated cells and unirradiated cells lost DiOC6 uptake (8 and 33% of DiOC6<sup>+</sup> for irradiated and unirradiated cells, respectively) (Fig. 5A, bottom).

The susceptibility of spleen cells from B6-/+ mice to exhibit disruption of mitochondrial transmembrane potential change is indicated by a decrease in DiOC6<sup>+</sup> positive cells in irradiated compared with unirradiated cells (Fig. 5B). There was a significant decrease in DiOC6<sup>+</sup> positive cells

![FIGURE 3. Phenotype-dependent resistance of sensitivity to γ-irradiation-induced apoptosis in B6-<sup>me/me</sup> spleen cells. Single-cell suspensions prepared from the irradiated (5 Gy) spleen cells of B6-<sup>me/me</sup> mice or B6-/+ mice were incubated at 37°C for 6 h. Cells were stained with the fluorochrome-conjugated anti-CD3 (T cells), anti-B220 (B cells), anti-CD11b (macrophages and granulocytes), or PE anti-CD5 plus FITC anti-CD3 (for the B cell population that was increased in B6-<sup>me/me</sup> mice) at room temperature for 20 min, then washed once with FACS buffer followed by 7-AAD staining. The results in each panel (7-AAD vs cell size) were obtained by gating the cells for the indicated cell population to differentiate the cell-specific sensitivity of γ-irradiation-induced apoptosis in the different cell types represented in the spleens of B6-<sup>me/me</sup> and control B6-/+ mice. The dot plot data is representative of results from four mice. The numerical data represents the percentage of apoptotic (7-AAD<sup>+</sup>) and nonapoptotic (7-AAD<sup>-</sup>) cells derived from four mice from each group analyzed individually.](http://www.jimmunol.org/)

---

### Table I. Increased percentage of cell survival rate after γ-irradiation of spleen cells from B6-<sup>me/me</sup> mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>CD3&lt;sup&gt;+&lt;/sup&gt; (T lymphocytes)</th>
<th>B220&lt;sup&gt;+&lt;/sup&gt; (B lymphocytes)</th>
<th>CD11b&lt;sup&gt;+&lt;/sup&gt; (Macrophages and Granulocytes)</th>
<th>CD3&lt;sup&gt;+&lt;/sup&gt; CD5&lt;sup&gt;+&lt;/sup&gt; (Abnormal B cells from B6-&lt;sup&gt;me/me&lt;/sup&gt; mice)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6-/+</td>
<td>36 ± 2%</td>
<td>38 ± 3%</td>
<td>54 ± 4%</td>
<td>52 ± 2%</td>
</tr>
<tr>
<td>B6-&lt;sup&gt;me/me&lt;/sup&gt;</td>
<td>56 ± 3%***</td>
<td>63 ± 4%***</td>
<td>68 ± 5%**</td>
<td>74 ± 4%*</td>
</tr>
</tbody>
</table>

---

* Single-cell suspensions were prepared from the spleen of B6-/+ or B6-<sup>me/me</sup> mice. The cells were irradiated at 5 Gy and were incubated at 37°C for 6 h before analysis for phenotype and cell survival rate by FACS as described in Materials and Methods. Results (Mean ± SE) indicated the percentage of cells that survived (7AAD<sup>-</sup>/large cells). There were four mice in each group.

*<sup>+</sup> *p* < 0.05; **<sup>+</sup> *p* < 0.01; ***<sup>+</sup> *p* < 0.005.
from irradiated B6-+/+ mice compared with unirradiated B6-+/+ mice at 6 and 24 h. In contrast, there was a minimal change in the ratio of DiOC6+ positive cells in irradiated compared with unirradiated cells from B6-me/me mice at 6 and 24 h.

Defective induction of Bax mRNA after γ-irradiation of B6-me/me spleen cells

The Bcl-2 gene family has been implicated in the regulation of the wide variety of mitochondrial events that are associated with stress-induced apoptosis. The gene products of members of this family, which include Bcl-2, Bax, Bcl-X 
, Bad, and Bak, can serve to either promote or inhibit apoptosis (27–29). Screening of the expression of the transcripts of these genes indicated a 3-fold increase (3.3 ± 0.2) in the expression of the Bax transcript in the spleen of B6-+/+ mice 3 h after γ-irradiation. In contrast, there was no significant increase in the expression (1.1 ± 0.2) of the Bax gene in the irradiated spleen cells of B6-me/me mice (p < 0.01, B6-+/+ vs B6-me/me) (Fig. 6, A and B). Although a lower level of induction of Bak transcription was observed in B6-me/me mice compared with normal mice after irradiation, the difference did not reach statistical significance. Only very low levels of Bcl-2 and Bad mRNA signals were detected in the spleen cells of both B6-+/+ and B6-me/me mice whether irradiated or not (data not shown), and there was no statistically significant difference in the expression of Bcl-2, Bcl-X 
, and Bad gene transcripts in B6-me/me cells compared with B6-+/+ cells. Moreover, 137Cs irradiation did not lead to a statistically significant change in the expression of Fas, FasL, Fas-associated death domain protein (FADD), or Fas-associated death domain-like IL-1-converting enzyme (FLICE; data not shown), suggesting that the induction of Fas-related death genes does not contribute significantly to the initiation of 137Cs irradiation-induced apoptosis.

Defective cell cycle block in B6-me/me cells after γ-irradiation

Tyrosine phosphorylation has been implicated as one of the major factors that determines whether cells undergo apoptosis or cell cycle arrest after γ-irradiation (30–32). To determine whether the resistance to apoptosis in the spleen cells of B6-me/me mice after γ-irradiation was associated with altered cell cycle kinetics, we used flow cytometry analysis of PI-stained cells (Fig. 7). Under steady-state conditions there was a significantly higher percentage of cells in the S + G2/M phase (22 ± 3%) and a decrease in the number of cells in the G0/G1 phase (73 ± 2%) in unirradiated B6-me/me spleen cells compared with the percentage of cells in these phases in the control unirradiated B6-+/+ cells (7 ± 1 and 89 ± 1%, respectively). There was defective cell cycle arrest in cells from B6-me/me mice, but not B6-+/+ mice, after γ-irradiation, indicated by the percentage of cells in the S + G2/M phases (31 ± 2 and 4 ± 4% for B6-me/me and B6-+/+, respectively, p < 0.005). There was a notable subG0 (hypodiploid or A0) peak in the histogram of B6-+/+ mice after γ-irradiation, whereas there was a lesser subG0 peak in the histogram of B6-me/me mice, indicating the presence of apoptotic cells in the B6-+/+ cell population and a decreased apoptosis in the B6-me/me cells. The average distribution of cells in different phases of the cell cycle in the unirradiated and irradiated B6-+/+ and B6-me/me spleen cells was calculated and is shown in Table II. These data show that the resistance to γ-irradiation-induced apoptosis in B6-me/me spleen cells is correlated with a decrease in the percentage of cells in the subG0 phase and an increase in the percentage of cells in the S + G2/M phase of the cell cycle.

FIGURE 4. Defective disruption of the mitochondrial transmembrane potential in B6-me/me spleen cells in response to γ-irradiation. The mitochondrial inner transmembrane potential collapse was determined by the exclusion of the fluorescent probe, DiOC 
, and evaluated microscopically using a DMS10 filter to visualize DiOC 
 staining of viable cells (green) and a UV-1A filter to visualize Hoechst-33342 staining of dead cells (blue). A and B, Results obtained for unirradiated B6-+/+ and B6-me/me cells, respectively. C, Spleen cells from a representative wt B6-+/+ mouse 6 h after γ-irradiation at 5 Gy. D, Spleen cells from a representative B6-me/me mouse 6 h after γ-irradiation at 5 Gy. E, Spleen cells from a representative wt B6-+/+ mouse 24 h after γ-irradiation at 5 Gy. F, Spleen cells from a representative B6-me/me mouse 24 h after γ-irradiation at 5 Gy.

Defective cell cycle block in B6-me/me cells after γ-irradiation

Tyrosine phosphorylation has been implicated as one of the major factors that determines whether cells undergo apoptosis or cell cycle arrest after γ-irradiation (30–32). To determine whether the resistance to apoptosis in the spleen cells of B6-me/me mice after γ-irradiation was associated with altered cell cycle kinetics, we used flow cytometry analysis of PI-stained cells (Fig. 7). Under steady-state conditions there was a significantly higher percentage of cells in the S + G2/M phase (22 ± 3%) and a decrease in the number of cells in the G0/G1 phase (73 ± 2%) in unirradiated B6-me/me spleen cells compared with the percentage of cells in these phases in the control unirradiated B6-+/+ cells (7 ± 1 and 89 ± 1%, respectively). There was defective cell cycle arrest in cells from B6-me/me mice, but not B6-+/+ mice, after γ-irradiation, indicated by the percentage of cells in the S + G2/M phases (31 ± 2 and 4 ± 4% for B6-me/me and B6-+/+, respectively, p < 0.005). There was a notable subG0 (hypodiploid or A0) peak in the histogram of B6-+/+ mice after γ-irradiation, whereas there was a lesser subG0 peak in the histogram of B6-me/me mice, indicating the presence of apoptotic cells in the B6-+/+ cell population and a decreased apoptosis in the B6-me/me cells. The average distribution of cells in different phases of the cell cycle in the unirradiated and irradiated B6-+/+ and B6-me/me spleen cells was calculated and is shown in Table II. These data show that the resistance to γ-irradiation-induced apoptosis in B6-me/me spleen cells is correlated with a decrease in the percentage of cells in the subG0 phase and an increase in the percentage of cells in the S + G2/M phase of the cell cycle.

FIGURE 5. Resistance to γ-irradiation-induced disruption of mitochondrial membrane potential in spleen cells of B6-me/me mice. A, Quantitation of the disruption of mitochondrial membrane potential was determined according to the reduction in DiOC6 uptake. Open areas represent the results from the unirradiated controls, and filled areas represent results for the irradiated cells. The numbers shown at the top of each representative histogram indicate the percentage of cells that stained DiOC6 positive in the irradiated compared with the unirradiated cells. B, A decrease in the uptake of DiOC6 in γ-irradiated spleen cells from B6-+/+ mice, but not B6-me/me mice. Results were presented as the percentage of DiOC6+ cells from unirradiated cells vs the γ-irradiated cells (*, p < 0.05; **, p < 0.01 between cells from B6-+/+ and B6-me/me mice). Cells from three mice were used in each group.
Dysregulated phosphorylation of p38 MAPK and p53 in γ-irradiated cells from B6-me/me mice and a SHP-1 mutant hybridoma cell line

To further investigate defective stress-activated signaling after γ-irradiation, the phosphorylation of several proteins that are involved in stress-induced response (including Erk1/2, p38 MAPK, JNK/SAPK, and p53) was examined in B6-+/+ and B6-me/me mice and in the BYDP hybridoma cell lines stably transfected with either wt SHP-1 or a dominant negative mutant form of SHP-1 (SHP-1 DΔP). There was very low expression of active phosphorylated p38 MAPK (P-p38 MAPK) 4 h after γ-irradiation of spleen cells from B6-me/me mice (Fig. 8A) or at all time points after γ-irradiation of the SHP-1 ΔΔP BYDP cell line (Fig. 8B). In contrast, there was up-regulation in the expression of phosphorylated p38 MAPK in control B6-+/+ mice and SHP-1 wt BYDP cells after γ-irradiation. There was no difference in the expression of phosphorylated Erk1/2 (P-Erk1/2; Fig. 8A) or JNK/SAPK (P-JNK/SAPK; Fig. 8, A and B) after γ-irradiation of spleen cells from B6-+/+ and B6-me/me mice or wt and SHP-1 ΔΔP BYDP cells. Interestingly, there was a markedly increased expression of the active phosphorylated form of p53 at 4 h after γ-irradiation of spleen cells from B6-me/me mice compared with B6-+/+ mice (Fig. 8A). These results suggest that the activation of p53 by γ-irradiation is not impaired in B6-me/me mice, and that defective phosphorylation of p38 MAPK is associated with the SHP-1 signaling defect in spleen cells from B6-me/me mice after γ-irradiation.

Discussion

Genotoxic stress response leading to DNA damage normally leads to a reversible cell cycle arrest allowing time for the cell to attempt to repair the DNA, followed by removal of cell cycle arrest upon successful completion of the repair (33, 34). The genotoxic stress response also can include the induction of apoptosis, which eliminates cells that have sustained irreparable DNA damage (35, 36). These processes ensure that the cells carrying DNA that has been damaged do not accumulate over the lifetime of individuals. Defects in these processes, resulting in either failure of the elimination of cells with damaged DNA or failure to adequately maintain the cells in cell cycle arrest, have been postulated to be key contributing factors in the development of cancer, autoimmune disease, and senescence. Indeed, such defects in the response to genotoxic stress have been reported to be the predominant characteristic of cells isolated from individuals with malignancies or autoimmune disease (37–42).

Kharbanda et al. (8) recently reported that SHP-1 is a substrate of c-Abl, a nuclear tyrosine kinase that plays an important role in regulating both cell growth and arrest depending on the cell type and environment (43, 44). The same investigators (8) further observed that phosphorylation of SHP-1 can occur in a c-Abl-dependent manner after treatment of cells with γ-irradiation in human U-937 myeloid leukemia cells. Because c-Abl can activate SAPK after γ-irradiation (8), this suggests the possibility that SHP-1 is an important intermediate between c-Abl and SAPK in human U-937 myeloid leukemia cells, and that SHP-1 might be an important molecule governing the cellular response to DNA damage in certain cells.

The results presented here provide the first evidence showing that a deficiency in SHP-1 is associated directly with defects in the genotoxic stress response to γ-irradiation in B6-me/me mice. The dose required to induce 50% cell death in spleen cells of B6-me/me mice was nearly 3-fold higher than that required for equivalent cell death in the spleen cells of wt B6-+/+ mice. This increased resistance to apoptosis was not due to a difference in the predominant cell types in B6-me/me mice compared with wt mice, as the muta
tion of SHP-1 increased the resistance of all major phenotypes of cells including T cells, B cells, CD11b+ macrophages and granulocytes, and CD3+ CD5+ cells, to γ-irradiation-induced apoptosis in B6-me/me mice. At a very high dose (>30 Gy), the B6-me/me spleen cells became sensitive to γ-irradiation-induced cell death. We also found that B6-me/me spleen cells were more sensitive to γ-irradiation-induced apoptosis compared with spleen cells from p53−/− mice (data not shown). Furthermore, there was a markedly
increased phosphorylation of p53 in spleen cell lysates from B6-me/me mice compared with wt mice after γ-irradiation. Thus, our results suggest that the activation of p53 was not impaired in spleen cells from B6-me/me mice after γ-irradiation and that SHP-1 is unlikely to be an upstream signal for the induction of the p53-mediated genotoxic apoptosis response.

High doses of ionizing radiation may induce massive cell death without leading to DNA repair mechanisms. Also, although cell death is maximal at 12 h after γ-irradiation, apoptosis signaling events precede the appearance of apoptotic cells. To study the mechanisms that contributed to the defective induction of the stress-activation response (apoptosis and cell cycle arrest) in spleen cells of B6-me/me mice after γ-irradiation, a moderate dose (5 Gy) of γ-irradiation and early time points (3- to 6-h) were chosen for this study because at the chosen dose and time point, there was a significant difference in sensitivity to γ-irradiation-induced stress response between cells from B6-me/me and wt mice. Based on the current observations, we predict that two mechanisms are involved in the defect in γ-irradiation-induced apoptosis in B6-me/me cells (Fig. 9).

A model for the role of SHP-1 in γ-irradiation-induced stress response. Our results suggest that two possible mechanisms are associated with the decreased sensitivity to γ-irradiation-induced apoptosis in spleen cells of B6-me/me mice. The first mechanism suggests that SHP-1 is an upstream signal for the induction of Bax and mitochondria transmembrane potential dissipation triggered by γ-irradiation. The second mechanism suggests that SHP-1 may be associated with p38 MAPK regulating cell cycle arrest through these two pathways.

Table II. Defective cell cycle block from B6-me/me mice after γ-irradiation

<table>
<thead>
<tr>
<th>Mice</th>
<th>Sub G0</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6+/+ (0Gy)</td>
<td>4 ± 1%</td>
<td>89 ± 1%</td>
<td>4 ± 1%</td>
<td>3 ± 0%</td>
</tr>
<tr>
<td>B6-me/me (0Gy)</td>
<td>5 ± 1%</td>
<td>73 ± 4**</td>
<td>14 ± 2*</td>
<td>8 ± 1**</td>
</tr>
<tr>
<td>B6+/+ (5Gy)</td>
<td>43 ± 5%</td>
<td>53 ± 4%</td>
<td>3 ± 3%</td>
<td>1 ± 1%</td>
</tr>
<tr>
<td>B6-me/me (5Gy)</td>
<td>15 ± 3***</td>
<td>54 ± 2%</td>
<td>20 ± 3***</td>
<td>11 ± 1***</td>
</tr>
</tbody>
</table>

* Single-cell suspensions prepared from wt B6-+/+ mice or Hcp1 mutant B6-me/me mice were either unirradiated or irradiated with γ-irradiation at 5 Gy and were harvested 6 h after the culture. Cells were stained with PI and analyzed by FACS analysis for cell cycle. Cell cycle analysis was carried out using cells derived from three mice in each group, analyzed individually (*, p < 0.05; **, p < 0.01; ***, p < 0.001; and between B6-+/+ and B6-me/me mice under the same treatment conditions).
and the collapse of the mitochondrial membrane potential. Taken together, these results suggest that SHP-1 plays an important role in γ-irradiation-induced apoptosis through the mitochondrial pathway regulated by Bax.

The second mechanism contributing to the reduced response to γ-irradiation-induced apoptosis in B6-me/me cells is a failure in the arrest of the cell cycle at the G1 phase. Our results showed that γ-irradiation induced a further expansion of cells in the S+G2/M phase in the cells of B6-me/me mice, rather than induction of a block in cell cycle entry. The abnormal cell cycle block in cells from B6-me/me mice after γ-irradiation could be due to linkage of SHP-1 signaling with cell cycle arrest induced by γ-irradiation through the c-Abi pathway (8). Yuan et al. (43, 44) reported that activation of c-Abi protein tyrosine kinase contributes to down-regulation of Cdk2 and cell cycle arrest at the G1 phase in response to genomic damage in a p53-dependent manner. We observed an increase in the phosphorylation of p53 from cell lysates of B6-me/me mice compared with wt mice both at steady state and particularly after γ-irradiation. Therefore, it is possible that a failure to induce G1 arrest in B6-me/me cells after γ-irradiation is associated with a failure to down-modulate a cyclin-dependent kinase, such as Cdk2, and that a constitutive phosphorylation of p53 may confer to an increased mitochondrial membrane potential changes in unirradiated cells from B6-me/me mice. Recently, Wang et al. (46) showed that activation of the p38 MAPK cascade was sufficient to induce cell cycle arrest in cells, and that expression of dominant negative alleles of MKK6 or p38γ allowed cells to escape the DNA damage-induced-G2 delay. The present experiments indicate that, after γ-irradiation, there was a decreased phosphorylation of p38 MAPK, but not Erk1/2 or SAPK/JNK, in spleen cell lysates from B6-me/me mice and lysates from SHP-1 mutant BYDP cells compared with the wt controls. Therefore, these results indicate that the decreased p38 MAPK phosphorylation is associated with a defective stress-activated response to γ-irradiation in spleen cells from B6-me/me mice.

In summary, our results show that mutation of SHP-1 is associated with defective cell cycle arrest and apoptosis induced by γ-irradiation; thus, functional SHP-1 could be acting as a tumor suppressor. As the homozygous me/me mice have a mean lifespan of 3 wk, their reduced ability to cope with environmental stress has not been recognized widely although their defects in hematopoiesis and the development of progressive fatal autoimmune syndromes have been studied extensively. Our results are consistent with the previous observation (1, 7) that Hcphme/me+ mice have a decreased tendency to develop lymphomas, although analysis of spleen cells from B6-Hcphme/me+ mice after γ-irradiation at 5 Gy showed a decreased, but not a significant, difference of their sensitivity to apoptosis compared with wt cells (data not shown). We postulated that the increased susceptibility to lymphoma in the B6-Hcphme/me+ heterozygous mice is due to a combined effect of both cell cycle defect and apoptosis defects after multiple DNA damage responses that occur over the lifetime of the mice, and thus was not detected after a single dose of γ-irradiation. Nevertheless, we (14, 15) and others (47) previously observed that SHP-1 is a signal transduction pathway that is involved in Fas- and acidification-mediation-mediated apoptosis in certain cells. Our present data lend further support to the concept that SHP-1 is a multifunctional protein and that it plays many of the different pathways that mediate apoptosis in response to different internal and external stimuli.

Acknowledgments

We thank Dr. Ulrike Lorenz at the University of Virginia for generously providing us the stable BYDP cell lines expressing either a wt or a mutant SHP-1. The FACS instrument was operated by Dr. T. Rogers and M. Spell of the FACS Core Facility at The University of Alabama at Birmingham. We also thank Dr. F. Hunter for critical review of the manuscript and Linda Flurry for excellent secretarial work.

References


