Replication Failure, Genome Instability, and Increased Cancer Susceptibility in Mice with a Point Mutation in the DNA Ligase I Gene

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ABSTRACT

DNA ligase I has a key role in DNA replication in the joining together of short replication intermediates. We used gene targeting to introduce a point mutation into the mouse DNA ligase I gene that was present in a human cancer patient with immunodeficiency and a cellular accumulation of DNA replication intermediates. Mutant mice grew more slowly and showed hematopoietic defects at critical stages at which the demands for DNA replication were highest. In the spleen and thymus of mutant mice, the accumulation of a sub-G0, but nonapoptotic, population was observed that we believe may represent cells with single-strand DNA breaks. In mutant bone marrow, occasional DNA replication failure was observed. The level of genome instability was significantly elevated in the spleens of DNA ligase I mutant mice and, because we have found no evidence for any DNA repair defect associated with DNA ligase I deficiency, we believe that this may result directly from the accumulation of replication intermediates. Mutant mice showed an increased incidence of spontaneous cancers with a diverse range of epithelial tumors, particularly cutaneous adnexal tumors that are rare in mice. The origin of the tumors from generalized genome instability, rather than the inactivation of one key control gene, should make DNA ligase I mutant mice a useful model to investigate the relationship between genome instability and cancer in humans.

INTRODUCTION

There are a number of inherited disorders that are associated with defects in DNA metabolism or DNA repair and that result in chromosome instability and cancer predisposition (reviewed in Ref. 1). The paradigm for genome instability disorders is BS, in which there is immunodeficiency, predisposition to a range of cancers, a very high level of sister chromatid exchange, increased mutation and somatic recombination rate, and increased sensitivity to a range of DNA-damaging agents (2). BS is attributable to mutations in the BLM (gene defective in BS) helicase gene (3).

Mutations in the DNA ligase I gene have been identified in a patient with similar growth retardation, immunodeficiency and photosensitivity as found in BS (4). A Glu-566 to Lys mutation at the active site inactivated one allele, whereas an Arg-771 to Trp mutation on the second allele resulted in 20-fold reduced enzyme activity (5). The 46BR cell line, isolated from this patient, showed increased sensitivity to DNA-damaging agents, delayed joining of Okazaki fragments, and elevated sister chromatid exchange, but not to the level seen in BS (6–9).

DNA ligase I is involved at the replication fork in the joining of Okazaki fragments during lagging strand DNA replication (10) and is considered the main replicative ligase. A correlation between DNA ligase I enzyme activity and the rate of cell proliferation supports this observation (11). DNA ligase I has also been implicated in NER (12, 13) and acts in the long-patch form of base excision repair (reviewed in Ref. 14). DNA ligase I has been reported to be an essential gene for mammalian cells growing in vitro (15).

We knocked out the mouse Lig1 gene to study the function of DNA ligase I and as the first stage in the production of a mouse model for the 46BR patient (16). As expected, the DNA ligase I mutation was an embryonic lethal, but mutant embryos developed much further than anticipated; they were indistinguishable from their siblings at embryonic development day 10.5 but then became progressively more anemic and died by embryonic development day 16.5. From embryonic development day 11.5 onwards, the liver takes over from the yolk sac as the major site of hematopoiesis. Mutant embryos failed to make this transition, with livers from mutant embryos lacking erythropoietic islands, leading to a severe deficiency of mature erythrocytes in the circulation. We found that hematopoietic progenitors were present in mutant fetal liver and that they were able to repopulate the hematopoietic system and rescue lethally irradiated recipients, which showed that there was not a qualitative hematopoietic block in Lig1 null cells, but rather that there was a quantitative impediment. We think that other tissues can compensate for the lack of DNA ligase I, presumably through the action of another ligase, but that this compensation fails under the high replicative pressure of fetal erythropoiesis, leading to reduced proliferation, anemia, and death. Using Lig1 null fibroblast cell lines, we found no evidence of any DNA repair deficit associated with DNA ligase I deficiency, but there was a delayed joining of DNA replication intermediates and decreased genome stability (17). Our work contradicts the suggestion (15) that Lig1 is essential for cell viability.

We have now carried out a further round of gene targeting to introduce the Arg-771-to-Trp mutation found in the 46BR patient into the mouse Lig1 gene. In this report, we describe the production of mice homozygous for the 46BR mutation and use the animals to study the relationship between DNA replication failure, genome instability, and cancer susceptibility.

MATERIALS AND METHODS

Production of Mice with the 46BR (Arg-771-to-Trp) Point Mutation in the Lig1 Gene. The 46BR-targeting vector consisted of the 13.5-kb EcoRI fragment containing exons 22–27 of the Lig1 gene, with the Arg-771 (CGG)-to-Trp (TGG) point mutation introduced into exon 23, cloned into the EcoRI site of pUC8. This is the same fragment used previously to make the Lig1 knockout vectors (16, 17). The point mutation was introduced in a PCR reaction for exon 23, using primers G3506 and G3507 and carried out on the cloned Lig1 gene fragment. Primer sequences were obtained from the mouse Lig1 cDNA sequence (Ref. 18; GenBank accession no. U19604) as follows: G3506, Forward, TTGGTGCCTACCTGGGCCGGGGGAAG; G3507, Reverse, CTGCTATCCGCGGCCGGGGGAAGTGGCGTCTGGCAGGGCG (a BamHI restriction site is underlined and the CGG-to-TGG point mutation is shown in bold and underlined); G3507, Reverse, CTGCTATCCGCGGCCGGGGGAAGTGGCGTCTGGCAGGGCG (a XmnI restriction site is underlined). The 101-bp PCR product containing the 46BR mutation was restricted at the naturally occurring BamHI and XmnI sites within exon 23 and cloned, via a series of intermediate steps, into the 13.5-kb EcoRI fragment in place of the wild-type sequence. The presence of the 46BR mutation and the lack of additional alterations in the exons (exons 22–27) present in the 46BR targeting vector was confirmed by DNA sequencing.
Two hundred μg of 46BR targeting vector DNA was restricted with EcoRI and electroporated as described (19) into 3 ∙ 10^7 HM-1 ES cells heterozygous for the Lig1- (no.12) knockout allele (17). Selection in 6-thioguanine was carried out as described previously (20), and resistant clones were picked and screened for the 46BR mutation. This was achieved by AcI restriction of a PCR product for exon 23 with primers MS175, Forward, CCCCAGATCGCTGGAGAAGGACTACCTTG (located at the 5′ end of exon 23 with an additional synthetic BamHI restriction site underlinded) and MS176, Reverse, CCGGAAATCCGCTCCATATAGCGTGAAGCTCTTCC (located at the 3′ end of exon 23 with an additional synthetic EcoRI restriction site underlinded). PCR was carried out as described (21), in 35 cycles with cycle conditions as follows: 94°C for 1 min, 63°C for 30 s, and 72°C for 30 s.

Clones heterozygous for the 46BR mutation were used for blastocyst injection and germ-line transmission was obtained as described (19). Germ-line chimeras were outbred with BALB/c and resulting 46BR homozygous lines and a wild-type control line were all maintained on a segregating 129/OlaXBALB/c background. Primary fibroblasts were isolated from mouse embryos on embryonic development days 10.5–13.5 and cultured as described previously (22).

Hematopoietic Cell Culture. Single-cell suspensions were obtained from bone marrow, spleen, and thymus by passing tissue progressively through finer needles down to 25-gauge. Cells were plated at 10^5/ml in 60-mm dishes in RPMI supplemented with 10% FCS, penicillin, and streptomycin. Proliferating spleenocyte cultures were also supplemented with lipopolysaccharides (25 μg/ml) and interleukin 4 (5 ng/ml). One h after plating, bone marrow and thymocyte cultures were irradiated with the medium with UV (254 nm; 10 J/m^2 for bone marrow, 20 J/m^2 for thymocytes). Proliferating spleenocyte cultures were treated with hydrogen peroxide (600 μM) in medium at 37°C for 30 min before replating into growth medium.

Flow Cytometry. Cell suspensions from hematopoietic tissues were prepared as described previously (16), counted electronically (Beckman Coulter), and diluted to 10^6/ml in PBS. Cells (10^6) were obtained from various suppliers: from Caltag Ltd., antimouse IgM F(ab′)_2, PE-conjugated affinity-purified goat IgG, antimouse CD4 PE-conjugated rat monoclonal IgG2a (clone CT-CD4), antimouse CD8α FITC-conjugated rat monoclonal IgG2a (clone CT-CD8a), antimouse B220 PE-conjugated rat monoclonal IgG2a (clone RA3-6B2), and antimouse Gr1 FITC-conjugated rat monoclonal IgG2b (clone RB6-8C5); from PharMingen, antimouse CD3 FITC-conjugated rat monoclonal IgG2b (clone 145-2C11), antimouse TCRαβ FITC-conjugated hamster monoclonal IgG (clone H57-597), and antimouse Ter-119 FITC-conjugated rat monoclonal IgG2b (clone Ter-119); and from Sigma-Aldrich Company Ltd., antimouse CD3 FITC-conjugated rat monoclonal IgG (clone 53-7.3). Propidium iodide-stained nuclei (20,000) were assayed on a Coulter EPICS XL to establish DNA content as described previously (22). Apoptotic cells were detected using the TACS Annexin V-FITC apoptosis detection kit using the manufacturer’s protocol (R&D Systems).

Detection of Micronuclei. Single-cell suspensions prepared from spleen and thymus were washed twice in PBS and fixed in absolute ethanol at −20°C. Fixed cells were stained with 4,6-diamidino-2-phenylindole (10 μg/ml). Fifteen μl of fixed cell suspension (10^6 cells/ml) were mounted on a slide, a coverslip added, and micronuclei were scored under a fluorescence microscope.

RESULTS

Production of Mice with the 46BR (Arg-771 to Trp) Point Mutation in the Lig1 Gene. The double-replacement gene-targeting strategy that we have devised for use with the Hprt-deficient ES cell line, HM-1, and Hprt minigenes as selectable markers (20, 23, 24) was used to introduce the human 46BR point mutation (Arg-771 to Trp) into the mouse Lig1 gene. The starting point was HM-1 cells heterozygous for the Lig1- (no.12) knockout allele (17). We have previously shown that the DWM110 Hprt minigenes present in this knockout allele is stably expressed, resulting in a very low frequency of spontaneous resistance to 6-thioguanine (25), which makes it ideal for the second step of double-replacement gene targeting.

The structures of the 3′ end of the wild-type Lig1 allele, the knockout allele, the 46BR-targeting vector, and the 46BR allele are shown schematically in Fig. 1. Homologous recombination between the knockout allele and the targeting vector will result in the incorporation of the 46BR mutation into the Lig1 locus, with selection for targeting events being provided by loss of the Hprt minigene and resistance to 6-thioguanine. Resistant clones were picked and 11 of the 38 that were screened contained the 46BR mutation. This was determined by AcI restriction of a PCR product for exon 23, the site of the 46BR mutation. The AcI recognition site (GCGG) coincides with the position of the 46BR mutation (GTCG) so that the Lig1-46BR allele is not cleaved. Seven clones were analyzed further by Southern blotting with a Lig1 cDNA probe (16) and found to be correctly targeted (data not shown). All of the clones had lost hybridizing restriction fragments diagnostic for the knockout allele and contained only fragments expected from the wild-type allele, with no evidence of randomly integrated vector sequences. The seven clones heterozygous for the 46BR mutation were used for blastocyst injection, and germ-line transmission was obtained from three. Founders were used to establish two independent lines homozygous for the 46BR mutation. The AcI polymorphism in exon 23 was used to genotype mice (see Fig. 1). Automated DNA sequencing of RT-PCR products was used as described previously (17) to confirm that the 46BR mutation was the only change present in the part of the Lig1 coding region (exons 22–27) involved in the gene-targeting manipulations.

46BR Mice Grow More Slowly. Mice homozygous for the 46BR mutation (46BR mice) developed normally and were overtly healthy.
However, from 5 weeks of age, it became apparent that they were growing more slowly than wild-type animals. This applied to both sexes (see Fig. 2 for males) and a 20% difference in body weight opened up and was maintained for up to 6 months, by which time the weights of wild-type animals had plateaued. When older animals (350–500 days) were sampled, it was found that the weights of 46BR mice had reached wild-type levels. These data are consistent with the DNA ligation defect associated with the 46BR mutation delaying DNA replication and slowing growth.

Normal Hematopoiesis in Older 46BR Mice. The hematopoietic tissues of 6- and 10-month-old 46BR mice were compared with age-matched controls for evidence of any defect. Hematocrits from 46BR mice were within the normal range (42–50%), and the number of cells recovered from all of the hematopoietic tissues examined (peripheral blood, bone marrow, mesenteric and peripheral lymph nodes, spleen, and thymus) were reduced only proportionately to the smaller size of the 46BR mice compared with controls. FACScan with antibodies to all hematopoietic cell lineages was used: B220 (expressed on cells committed to the B lymphoid lineage); IgM and IgD (present on mature B lymphocytes which have undergone V(DJ) recombination); CD4 and CD8 (present on thymocytes); TCRβ (present on thymocytes with rearrangement of the TCR gene); CD3 (present on T cells and a subset of B cells); CD45 (present on nonerythroid hematopoietic cells); GR1 (present on granulocytes); and Ter-119 (present on erythroid cells). The staining profiles of 46BR null embryos (16) and in lethally irradiated mice rescued by \( \text{Lig1} \) null embryos (17) and in lethally irradiated mice rescued by \( \text{Lig1} \) null embryos (17) are the same as those of wild-type embryos (17).

Fig. 2. 46BR mice grow more slowly than wild-type controls. The relationship between body weight and age is shown for male wild-type (●) and 46BR (□) mice. Mice (47 wild-type and 29 46BR animals) were weighed on 2–3 separate occasions to obtain the data shown. Each data point, the mean weight from two or more animals.

46BR littermates sampled on consecutive days. In the wild-type spleens, the frequency of S-phase cells varied from 4 to 9% (Fig. 4, A, C, and E). In the 18-day-old 46BR spleen, 25% of the cells were in S phase (Fig. 4B). This high frequency of S-phase cells was maintained

![Image](image-url)

Table 1. The frequency of S-phase cells in tissues from wild-type and 46BR micea

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Wild-type</th>
<th>46BR normal</th>
<th>46BR abnormalb</th>
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<tbody>
<tr>
<td>Spleen (19–23 days)</td>
<td>8.2 ± 1.1 (n = 11)</td>
<td>19.1 ± 1.3 (n = 11)</td>
<td>ND</td>
</tr>
<tr>
<td>Spleen (5–14 wk)</td>
<td>2.6 ± 0.2 (n = 12)</td>
<td>2.9 ± 0.4 (n = 12)</td>
<td>ND</td>
</tr>
<tr>
<td>Bone marrow (3–14 wk)</td>
<td>13.3 ± 0.8 (n = 10)</td>
<td>18.3 ± 0.5 (n = 10)</td>
<td>7.8 ± 0.5 (n = 3)</td>
</tr>
<tr>
<td>Thymus (3–14 wk)</td>
<td>5.4 ± 0.3 (n = 10)</td>
<td>6.3 ± 0.2 (n = 10)</td>
<td>6.3 ± 0.3 (n = 10)</td>
</tr>
</tbody>
</table>

a The mean frequency of S-phase cells is expressed as a percentage of the total number of cells assayed (>20,000 cells for all samples). The SE and the number of individual mice sampled (n) are shown.

b Flow cytometry profiles having an abnormal appearance, either a discrete sub-G1 peak or DNA replication failure. Four % of 46BR spleen (see Fig. 4), 7% of bone marrow, and 27% of thymus (see Fig. 5) samples fell into this category.

c ND, not determined; P, probability by Student’s t test for the null hypothesis that the frequency of S-phase cells is the same for each 46BR sample as it is for its wild-type control.
in a 19-day-old littermate, but there was now also a prominent and discrete sub-G₁ peak comprising 21% of the cells (Fig. 4D). This discrete sub-G₁ peak was seen in 4% (3 of 73) of 46BR spleens examined but was not observed in wild-type spleens (0 of 45 examined). The explanation for this sub-G₁ peak will be considered later.

Bone marrow samples taken from four individual 19- and 20-day 46BR mice did not show the same marked increase in the frequency of S-phase cells that was observed in spleen. However, a slight, but significant, increase in the frequency of S-phase cells was found when flow cytometry profiles from 3–14-week-old 46BR bone marrows were compared with wild-type controls (Table 1). Although the large majority of 46BR bone marrows examined had profiles like the wild-type control sample shown in Fig. 5A, 7% (3 of 45 examined) had an abnormal profile like the example in Fig. 5B. In these abnormal bone marrows, the frequency of S-phase cells was 2-fold less than in normal 46BR samples (Table 1). This difference between abnormal and normal 46BR bone marrow was significant (P = 2.6 × 10⁻⁷), as was the lower frequency of S-phase cells in abnormal 46BR bone marrow compared with wild-type controls (see Table 1). In the abnormal 46BR bone marrows, there was no longer a discrete G₂ peak corresponding to cells that had completed S phase. In the normal 46BR sample shown (Fig. 5B), only 2% of the cells are in G₂ compared with 4% of the wild type (Fig. 5A) and a smaller fraction of the population (8%) is in S phase compared with the wild type (14%).

The abnormal profile of altered replication seen in some bone marrow samples from 46BR mice in vivo was very different from the typical profile associated with DNA-damage-induced apoptosis in vitro. Bone marrow was prepared from wild-type animals, and one-half was used for propidium iodide staining of nuclei to confirm the presence of a discrete apoptotic population (23%) of Annexin V and propidium iodide double-labeled cells (Fig. 5D). Similar staining of normal 46BR and wild-type bone marrows did not reveal an increased frequency of cells in the late stages of replication.

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DNA Ligase I-DEFICIENT MICE

Fig. 5. Altered DNA replication and a discrete sub-G1 peak in hematopoietic tissues from 46BR mice. A, B, C, E, and G, the frequency distribution of DNA content from propidium iodide-stained nuclei obtained by flow cytometry. D, F, and H, Annexin V and propidium iodide labeling of live cells by flow cytometry. Cells (20,000) were assayed for DNA content of propidium iodide-stained nuclei 18 h after a UV dose of 10 Jm\(^{-2}\); sub-G1, 19%; G1, 75%; S phase, 4%; G2 1%. The classic G1 arrest and broad apoptotic sub-G1 peak are notable. D, Annexin V and propidium iodide labeling of live cells sampled from the same experiment shown in C. A discrete population (23%) of double-labeled apoptotic cells (quadrant 2) is present. E and F, thymus sample from a 4-week-old thymocyte. E, sub-G1 1%, G1 59%, S phase 8%, G2 4%; F, 3% double-labeled apoptotic cells. G and H, thymus sample from 8-week-old 46BR mouse. G, sub-G1 30%, G1 59%, S phase 8%, G2 2%; H, 3% double-labeled apoptotic cells. The discrete, large sub-G1 peak in the 46BR thymus sample is not accompanied by a high level of apoptosis. Arrow, a small peak at twice the DNA content of the sub-G1 peak.

level of apoptosis in 46BR samples (data not shown). The apoptosis assay has not been possible on abnormal 46BR bone marrows because of the low frequency of occurrence.

The Sub-G1 Peak in Thymus from 46BR Mice Is Not Caused by Apoptosis. The majority of flow cytometry profiles from 3–14-week-old 46BR thymuses were normal, but there was a small, but significant, increase in the frequency of S-phase cells compared with wild-type controls (Table 1). However, 27% (19 of 70) of 46BR thymuses examined had abnormal profiles with a pronounced discrete sub-G1 peak (Fig. 5G). In the example shown, 30% of the population is in the sub-G1 peak. The frequency of S-phase cells in these abnormal 46BR thymuses was the same as in normal 46BR thymuses (Table 1). In abnormal 46BR thymuses with the most pronounced sub-G1 peaks, there was also a small peak corresponding to double the DNA content of the sub-G1 peak (Fig. 5G, arrow). These discrete sub-G1 peaks were not observed in wild-type thymuses (0 of 39 examined).

These data are consistent with the ligation defect associated with the 46BR mutation having a detrimental effect on DNA replication. In some bone marrows, the effect is severe, leading to a failure of normal completion of DNA replication. The obvious explanation for the discrete sub-G1 peak in 46BR thymuses, namely, that it was caused by apoptosis triggered by the replication defect, was ruled out by Annexin V and propidium iodide double-labeling of live cells. The frequency of double-labeled apoptotic cells was the same (only 3%) in wild-type (Fig. 5F) and abnormal 46BR thymus (Fig. 5H), despite the presence in the latter of 30% of the population in a discrete sub-G1 peak.

The Sub-G1 Peak Can Be Induced in Wild-Type Hematopoietic Cell Cultures by DNA-damaging Agents. We have shown that the discrete sub-G1 peak that occurs spontaneously in 46BR thymus is not caused by apoptosis. To understand how it could arise we sought to induce a similar peak in wild-type hematopoietic cell cultures. Wild-type thymocyte cultures were established and either UV-irradiated at 20 Jm\(^{-2}\) or left untreated. Eighteen h later, control cultures showed a broad sub-G1 peak (9% of the population in Fig. 6A), that correlated well with the frequency of apoptotic cells (14%; Fig. 6B). As expected, the frequency of apoptotic cells was increased in cultures 18 h after UV-irradiation (20%, Fig. 6D; 25%, Fig. 6F). Some irradiated cultures (Fig. 6C) showed the same broad sub-G1 peak as control cultures, whereas others (Fig. 6E) displayed, in addition, a discrete sub-G1 peak reminiscent of that seen in abnormal 46BR thymuses in vivo. The equivalent levels of apoptosis in the two types of irradiated culture indicated that it was the broad, rather than the discrete, sub-G1 peak that contained apoptotic cells. The effect of a second DNA-damaging agent, hydrogen peroxide, was studied in proliferating wild-type spleenocyte cultures. Control spleenocyte cultures had a higher frequency of S-phase cells and of the broad sub-G1 peak, diagnostic for apoptosis, than unstimulated control thymocytes (spleenocytes, Fig. 6G: sub-G1, 17%; S phase, 9%; thymocytes, Fig. 6A: sub-G1, 9%; S phase, 1%). Treatment with hydrogen peroxide (600 μM for 30 min) did not affect the frequency of S-phase cells, but 24 h after treatment, the frequency of cells in the sub-G1 peak increased, and a discrete peak superimposed on the broad sub-G1 peak of apoptotic cells was visible. This discrete sub-G1 peak became more pronounced 2 days after treatment (Fig. 6H). Thus, the characteristic discrete sub-G1 peak, seen spontaneously in 46BR spleens and thymuses in vivo, can be reproduced by treatment of wild-type spleen and thymus cultures in vitro with DNA-damaging agents.

46BR Mice Show Increased Genome Instability. We have previously shown that primary fibroblasts isolated from Lig1 null mouse embryos have a 4-fold higher frequency of micronuclei than wild-type control cultures (17). To investigate whether the DNA ligase I deficiency associated with the 46BR mutation also caused an increased level of genome instability, we first isolated 46BR primary embryonic...
Fig. 6. The discrete sub-G1 peak can be induced in wild-type hematopoietic cell cultures by DNA-damaging agents. A, C, E, G, and H, the frequency distribution of DNA content for propidium iodide-stained nuclei obtained by flow cytometry. Cells (20,000) were assayed for each sample, and the results for each cell type are displayed on the same frequency scale. A and B, control thymocyte culture. A, sub-G1 9%, G1 87%, S phase 1%, G2 3%. B, 14% double-labeled apoptotic cells. C and D, thymocyte culture 18 h after UV-irradiation. C, sub-G1 16%, G1 79%, S phase 3%, G2 2%. D, 20% double-labeled apoptotic cells. E and F, independent thymocyte culture 18 h after UV-irradiation. E, sub-G1 51%, G1 45%, S phase 3%, G2 1%. F, 25% double-labeled apoptotic cells. The presence of the discrete sub-G1 peak in this irradiated sample, which is independent of the frequency of apoptosis, is notable. G, proliferating control spleenocyte culture; sub-G1 16%, G1 72%, S phase 9%, G2 3%. H, proliferating spleenocyte culture 2 days after hydrogen peroxide treatment; sub-G1 29%, G1 55%, S phase 12%, G2 4%. The presence of the discrete sub-G1 peak in the treated sample is notable.

Table 2  The frequency of micronuclei in tissues from wild-type and 46BR mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Tissue</th>
<th>Age</th>
<th>Micronuclei (%)</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>Primary fibroblasts</td>
<td>Passage 2–4</td>
<td>0.49 ± 0.21 (n = 6)</td>
</tr>
<tr>
<td>46BR</td>
<td>Primary fibroblasts</td>
<td>Passage 2–4</td>
<td>0.47 ± 0.15 (n = 4)</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>7 wk</td>
<td>0.15 ± 0.10 (n = 4)</td>
</tr>
<tr>
<td>Wild type</td>
<td>Thymus</td>
<td>7 wk</td>
<td>0.25 ± 0.07 (n = 4)</td>
</tr>
<tr>
<td>46BR</td>
<td>Thymus</td>
<td>7 wk</td>
<td>0.51 ± 0.13 (n = 4)</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>7 wk</td>
<td>0.94 ± 0.13 (n = 4)</td>
</tr>
<tr>
<td>Wild type</td>
<td>Spleen</td>
<td>15 mo</td>
<td>0.32 ± 0.11 (n = 6)</td>
</tr>
<tr>
<td>46BR</td>
<td>Spleen</td>
<td>15 mo</td>
<td>0.51 ± 0.29 (n = 7)</td>
</tr>
<tr>
<td></td>
<td>Primary fibroblasts</td>
<td>Passage 2–4</td>
<td>0.48 ± 0.30 (n = 6)</td>
</tr>
<tr>
<td>46BR</td>
<td>Spleen</td>
<td>15 mo</td>
<td>1.60 ± 0.45 (n = 7)</td>
</tr>
</tbody>
</table>

a Primary embryonic fibroblasts from embryonic development day 13.5 wild-type and 46BR embryos and cells prepared from the spleens and thymuses of wild-type and 46BR mice were scored for micronuclei. For each sample, >1000 cells were scored. The mean frequency of micronuclei is expressed as a percentage of the number of cells scored. The SD and the number of individual animals sampled (n) is also shown.

b P, by Student’s t test for the null hypothesis that the frequency of micronuclei is the same for each 46BR sample as it is for its wild-type control.

DISCUSSION

Role of DNA Ligase I. DNA ligase I has a key role in DNA replication in the joining of Okazaki fragments during lagging strand
DNA synthesis (10, 26–28). In vitro reconstitution experiments have also shown that DNA ligase I is involved in NER and the long-patch form of base excision repair (12–14). Thus, the report that DNA ligase I was a cell-essential gene (15) was not unexpected. We have generated two different Lig1 knockout alleles and shown both to be embryonic lethals (16, 17). However, mutant embryos from both knockout lines developed normally to midterm, and viable cell lines were isolated from the embryos. This clearly contradicts the assertion that Lig1 is a cell-essential gene. Mutant embryos failed with a specific defect in erythropoiesis, although hematopoietic progenitors from Lig1 null fetal liver were able to repopulate the hematopoietic system and rescue lethally irradiated recipients. We believe that other tissues can compensate for the lack of DNA ligase I, presumably through the action of another ligase, but that this compensation fails under the high replicative pressure of fetal erythropoiesis, leading to reduced proliferation, anemia, and death.

We found no evidence for any DNA repair defect in Lig1 null cells, but an accumulation of DNA replication intermediates and increased genome instability were detected (17). For this reason, we consider that failed erythropoiesis in Lig1 null embryos, increased genomic instability in Lig1 null cells, and the phenotype in the human 46BR patient with point mutations in the Lig1 gene (see next section) are likely to result directly from the DNA replication defect, although we cannot exclude the possibility that an as-yet-undetected DNA repair deficiency may also be involved.

Mice Homozygous for the Arg-771-to-Trp Mutation in the Lig1 Gene as a Model for the 46BR Patient. Two different DNA ligase I point mutations have been reported in a single patient (4) with similar growth retardation and immunodeficiency as found in BS, a paradigm for inherited genome instability/cancer predisposition diseases (2). This 46BR patient developed lymphoma at age 17 and subsequently died from pneumonia. A Glu-566-to-Lys mutation at the active site inactivated one allele, whereas an Arg-771-to-Trp mutation on the second allele resulted in 20-fold reduced enzyme activity (5). We have used double-replacement gene targeting in the Hprt-deficient ES cell line, HM-1, to generate mice homozygous for the Arg-771-to-Trp mutation. We chose this milder mutation because only the Arg-771-to-Trp allele is retained in the 46BR cell line. The Glu-566-to-Lys allele, encoding a completely nonfunctional protein, has been lost, and we reasoned that this mutation, like our Lig1 knockouts, would probably be an embryonic lethal in mice. Mice homozygous for the Arg-771-to-Trp mutation are, thus, not a perfect model for the 46BR patient. We also have the potential to generate mice heterozygous for the 46BR mutation over a knockout allele, differences in the phenotype of these two models (Lig1^{46BR/46BR} and Lig1^{46BR/-}) might be anticipated.

Slower Growth but Normal Hematopoiesis in Older 46BR Mice. From 5 weeks onward, 46BR mice grew more slowly than wild-type controls and took longer to attain the same mature body weight. This was consistent with their growth being slowed by the DNA ligase I defect leading to the accumulation of DNA replication intermediates. There was no evidence for a spontaneous hematopoietic defect in the older 46BR mice that might have been predicted from the immunodeficiency of the 46BR patient. All hematopoietic lineages were present in the expected amounts. However, the mice were housed in a clean environment, and their health status was good so that the ability of their immune system to respond to infection was not being tested. A difference in the ability of the hematopoietic systems of 46BR and control mice to respond might become apparent in more challenging circumstances.

Erythropoiesis Is under Stress in Young 46BR Mice. We have previously identified erythropoiesis in Lig1 null embryos and, after transplantation of Lig1 null fetal liver cells into lethally irradiated recipients, as being critical situations in which Lig1 null cells are unable to meet the particularly high demand for DNA replication (16, 17). The enlargement of the spleen in 3-week-old 46BR mice, the elevated frequency of S-phase cells, and the increased frequency of immature reticulocytes released prematurely into the circulation, all indicate that erythropoiesis is facing a similar but less severe difficulty in meeting replicative demands attributable to the DNA ligase I deficiency. Alternatively, if an as-yet-undefined DNA repair deficiency contributes to the phenotype of 46BR mice, then a higher exposure to the relevant damage in spleen might also explain this result. This situation quickly resolved, presumably because the demand for red cells dropped, or because of the increasing number of hematopoietic cells that can be devoted to erythropoiesis, and by 5

Fig. 7. Reduced tumor-free survival in 46BR mice. Animals (34 wild-type and 53 46BR) were kept for up to 2 years and the incidence of spontaneous tumors was recorded. Seventeen tumors were recorded in the 46BR mice: lymphoma (8), adenocarcinoma (3), mixed adenocarcinoma (2), bronchoalveolar carcinoma (1), cystadenoma (1), hiliar cell tumor (1), hemangioma (1). Two tumors were found in wild-type mice: adenocarcinoma (1), osteosarcoma (1).

Fig. 8. Adnexal tumor from a 46BR mouse. H&E-stained sections of a skin adnexal tumor. A, intermediate-power view (×100) showing the lobular architecture with basalloid cells forming ductular profiles (D) and showing abrupt zonal keratinization (K). B, higher power view (×400) showing brisk mitotic activity (arrowheads) and necrosis (N) within ductular lumina.
weeks of age, the sizes of the spleens and the level of DNA replication in 46BR mice were indistinguishable from those in wild-type controls.

**Occasional Failure of DNA Replication in Hematopoietic Tissue from 46BR Mice.** Apart from the 2-fold increase in the frequency of S-phase cells in the spleens from 3-week-old 46BR mice, thymus and bone marrow samples also showed a smaller but significant increase. We believe that this reflects an increased number of cells in S phase, because replication takes longer to complete because of the ligase deficiency, rather than an increased rate of proliferation. In rare samples from 46BR bone marrow, the frequency distribution of nuclear DNA content was consistent with a failure occurring during DNA replication, again presumably because there was insufficient DNA ligase I activity in the 46BR bone marrow to sustain the required high rate of proliferation. This in vivo frequency distribution in 46BR bone marrow was quite distinct from that seen after the induction of apoptosis by DNA damage in wild-type bone marrow in vitro, which was associated with a classic G1 arrest, and the prevention of initiation of DNA replication as opposed to the apparent failure during DNA replication seen in vivo. Indeed, although we have observed spontaneous prominent sub-G1 peaks in 46BR spleen and thymus in vivo, we have shown clearly that this is not caused by apoptosis. Thus, we have no evidence that apoptosis is acting to remove abnormal hematopoietic cells that accumulate in 46BR mice because of the DNA ligase deficiency.

The **Discrete Sub-G1 Peaks in 46BR Spleen and Thymus Could Arise through Accumulation of Single-Strand DNA Breaks.** Although broad sub-G1 peaks represent apoptotic cells, we have clearly shown that the discrete sub-G1 peaks in 46BR spleen and thymus are not caused by apoptosis. How then can they be explained? Aneuploidy is not a plausible alternative because the sub-G1 peaks indicate apparent reductions in DNA content of up to 35% (32% in the 46BR thymus shown in Fig. 5B). Such cells would be inviable and unable to replicate their DNA in the way that the abnormal cells in 46BR thymus appear able to do, if our interpretation of the small peak at twice the sub-G1 peak value in samples such as that shown in Fig. 5B is correct. Such discrete sub-G1 peaks can also appear rapidly (overnight after DNA damage in wild-type cultures) and may be relatively persistent in vivo. Although we are unable to sample any single animal twice, the presence of discrete sub-G1 peaks in the thymus of 46BR littermates sampled 2 weeks apart indicates that such peaks may be long-lasting. We consider that only one possible explanation remains: that the sub-G1 peak represents an apparent, rather than a real, reduction in DNA content. Such a change could arise from single-strand DNA breaks reducing DNA supercoiling and leading to a reduction in the amount of propidium iodide staining. The amount of propidium iodide fluorescence in flow cytometry is dependent on the method of sample preparation (29), and the fluorescence from such intercalating dyes is sensitive to the degree of DNA supercoiling, although, in the case of the related dye ethidium bromide, removing supercoiling actually increases the amount of dye that can bind (30). An increased number of single-strand DNA breaks would be predicted in 46BR mice from the accumulation of DNA replication intermediates that we have detected in Lig1 null cells (17), or from an, as-yet-undetected DNA repair deficiency. This argument is strengthened by our observation that we can induce equivalent sub-G1 peaks in wild-type cultures in vitro with DNA-damaging agents. Single-strand breaks will be produced during NER of UV-induced damage, whereas hydrogen peroxide causes strand breaks directly, in addition to a range of other lesions. This hypothesis for the origin of the discrete sub-G1 peak in 46BR mice can be tested by measuring single-strand breaks directly by end-labeling or using the alkaline comet assay. In addition to the rate of proliferation in a particular tissue, the other factor that may govern the appearance of the discrete sub-G1 peak in 46BR mice is the level of expression of DNA ligase I and of the other ligases, which may in some circumstances be able to compensate for ligase I deficiency (17). If our interpretation is correct, we predict that we could increase the frequency of such events by stressing the hematopoietic system, either by blocking DNA replication and then releasing it to generate a surge of replication or by raising a strong, prolonged polyclonal immune response.

**Genomic Instability in 46BR Mice Results from Abnormal DNA Replication.** We have previously observed an increased frequency of genome instability, assayed by micronucleus formation, in primary fibroblasts isolated from Lig1 null embryos (17). It is not unreasonable to suppose that strand breaks and replication failure might result in increased genome instability in 46BR mice. Because DNA repair functions were normal in Lig1 null cells, we consider that the genomic instability is more likely to result directly from the accumulation of replication intermediates rather than from an as-yet-undetected DNA repair deficiency, and we favor a similar explanation for the increased levels of instability observed in 46BR mouse tissues in vivo, in which micronucleus formation was 3-fold higher in spleens from 15-month-old 46BR mice than from controls. The failure to observe an increase in genomic instability in 46BR primary embryonic fibroblasts in vitro that is similar to what we reported in Lig1 null cells could be caused by the 46BR allele encoding a protein with some residual ligase I activity. The difference between the in vivo and in vitro results for mouse 46BR cells could arise from the greater constraints on the coordination of DNA replication and mitosis in a tightly regulated process such as hematopoiesis in vivo. Pressure to enter mitosis to a precise schedule in vivo but not in vitro, coupled with a delay in replication arising from the accumulation of replication intermediates, could result in a failure of chromosome segregation or the loss of chromosome fragments, which would both generate micronuclei.

**Increased Susceptibility to Cancer in 46BR Mice.** Genome instability is a key event in the development of cancer. The modest but significant increase in instability observed in the spleens of 46BR mice correlates well with the modest increase in the spontaneous tumor incidence observed. Thirty-two% of our 46BR mice developed tumors over a 2-year study, compared with only 6% of controls. Our model for the origin of these cancers is as follows: the accumulation of replication intermediates in cells in which the reduced amounts of DNA replication as opposed to the apparent failure during DNA replication seen in vivo, which is micronucleus formation was 3-fold higher in spleens of 15-month-old 46BR mice than from controls. The failure to observe an increase in genomic instability in 46BR primary embryonic fibroblasts in vitro that is similar to what we reported in Lig1 null cells could be caused by the 46BR allele encoding a protein with some residual ligase I activity. The difference between the in vivo and in vitro results for mouse 46BR cells could arise from the greater constraints on the coordination of DNA replication and mitosis in a tightly regulated process such as hematopoiesis in vivo. Pressure to enter mitosis to a precise schedule in vivo but not in vitro, coupled with a delay in replication arising from the accumulation of replication intermediates, could result in a failure of chromosome segregation or the loss of chromosome fragments, which would both generate micronuclei.

**Occurrence of Cutaneous Adnexal Tumors in 46BR Mice.** We have observed two of these unusual tumors in our current study of 46BR mice and an additional six in an ongoing study. No wild-type control animals in these studies have developed any such adnexal neoplasm. Cutaneous adnexal tumors, especially carcinomas, are rare neoplasms both in humans and in the mouse, and precise classification remains the object of discussion. Although the designations “basosquamous tumor,” of both benign and malignant types, have been applied to the mouse, this nomenclature is considered inappropriate for the tumors under discussion in this study, given their histological appearance with convincing evidence of ductular differentiation and the rather abrupt and, frequently, very prominent pilar-type keratinization. Although most commonly re-
The existing mouse models for cancer do not always accurately reflect human cancer because they are not as susceptible to cancer as humans. For example, some mouse models have a higher incidence of cancer than humans, while others have a lower incidence. This discrepancy may be due to differences in the genetic makeup of humans and mice, as well as differences in the environment in which they are raised.

**Comparison with Other Genetically Manipulated Mouse Models of Cancer Susceptibility.** Existing genetically manipulated mouse models for cancer susceptibility involve changes to key genes that act to prevent or cause cancer, such as inactivation of tumor suppressor genes (reviewed in Ref. 33), inactivation of DNA repair genes (reviewed in Ref. 1), or activation of oncogenes such as the ras family (reviewed in Ref. 34). Typically, the resulting increases in cancer susceptibility are dramatic (e.g., for the inactivation of the p53 gene, 74% of animals developed tumors within 6 months; Ref. 35), reflecting the importance of the manipulated gene to carcinogenesis, but there is often a strong bias in the types of tumor seen. Such genetically modified mice, in which the alteration is present in every cell, do not represent good models for the majority of human cancers that originate from sporadic genetic alterations in individual cells surrounded by normal neighbors. This discrepancy has been addressed in an improved model for sporadic cancer involving activation of a mutant K-ras gene in gene-targeted mice when intrachromosomal recombination occurs randomly in individual somatic cells (36). If we are correct in our interpretation of the mechanism for carcinogenesis in 46BR mice, this would represent a very different model of cancer susceptibility, one in which the increased cancer frequency results indirectly from increased genome instability rather than directly from the change in expression of a key gene involved in carcinogenesis. As such, this would more closely model the situation in sporadic human cancers that can arise in a wide range of cell types and tissues. The benefits of such a model would be that, potentially, any type of tumor could be obtained, provided that the cell type involved was undergoing DNA replication. The disadvantage is that the tumor frequency, although higher than in control mice, could never be as high as in models in which specific genes directly involved in carcinogenesis have been manipulated. The diverse range of spontaneous tumors seen in our first survey of 46BR mice, particularly the occurrence of a number of unusual epithelial tumors rarely seen in mice, indicates that mice with point mutations in the DNA ligase I gene will, indeed, prove to be a valuable addition to the existing mouse models for cancer.

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**REFERENCES.**


**DNA Ligase I-DEFICIENT MICE.**

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Replication Failure, Genome Instability, and Increased Cancer Susceptibility in Mice with a Point Mutation in the DNA Ligase I Gene


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