Haploinsufficiency in DNA Polymerase β Increases Cancer Risk with Age and Alters Mortality Rate

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Abstract

This study uses a base excision repair (BER)–deficient model, the DNA polymerase β heterozygous mouse, to investigate the effect of BER deficiency on tumorigenicity and aging. Aged β-pol$^{+/-}$ mice express 50% less β-pol transcripts and protein ($P < 0.05$) than aged β-pol$^{+/+}$ mice, showing maintenance of the heterozygous state over the life span of the mouse. This reduction in β-pol expression was not associated with an increase in mutation rate but was associated with a 100% increase in the onset of hypoploidy. Aged β-pol$^{+/-}$ mice exhibited a 6.7-fold increase in developing lymphoma ($P < 0.01$). Accordingly, 38% of β-pol$^{+/-}$ mice exhibited lymphoid hyperplasia, whereas none of the β-pol$^{+/+}$ exhibited this phenotype. β-pol$^{+/-}$ mice were also more likely to develop adenocarcinoma (2.7-fold increase; $P < 0.05$) and more likely to develop multiple tumors, as 20% of the β-pol$^{+/-}$ animals died bearing multiple tumors compared with only 5% of the β-pol$^{+/+}$ animals ($P < 0.05$). In spite of accelerated tumor development, no gross effect of β-pol heterozygosity was seen with respect to life span. However, the survival curves for the β-pol$^{+/+}$ and β-pol$^{+/-}$ mice are not identical. A maximum likelihood estimation analysis showed a modest but significant ($P < 0.05$) acceleration of the age-dependent mortality rate in β-pol$^{+/-}$ mice. Thus, the β-pol$^{+/-}$ mouse represents a model in which mortality rate and tumor development are accelerated and provides evidence supporting the role of genomic maintenance in both aging and carcinogenesis. (Cancer Res 2006; 66(15): 7460-5)

Introduction

Loss of base excision repair (BER) increases genomic instability (1), which has been implicated as a causal factor in both cancer and aging (2, 3). Gene products that play a role in DNA repair are likely to be important factors determining these processes. In several well-characterized syndromes of premature aging, loss of genome maintenance genes have been described (3), supporting the role that DNA repair plays in protection from the aged phenotype. DNA repair mechanisms seem to be designed to maintain the genome through the reproductive period, thus protecting the germ line. Subsequently, DNA repair capacity deteriorates (4, 5), giving rise to age-related acceleration of genomic instability. Additional losses of repair capacity caused by genetic defects, or by targeted disruption of a DNA repair gene in mouse models, should be expected to further accelerate this rate of decline, thus increasing the incidence of tumors. The role for DNA repair in preventing tumor development is clear in that defects in a variety of DNA repair pathways, especially nucleotide excision repair, can greatly increase cancer risk (6).

The role for the BER pathway in these processes has been less obvious. In part, this arises from the embryonic lethality of null mutations in the BER core genes, including *AP endonuclease, XRCC1*, and *DNA polymerase β* (*β-pol*). However, it is also difficult to identify loss of BER as a critical factor in carcinogenesis because BER and β-pol are essential in the repair of endogenously arising base damage. This makes it nearly impossible to identify potential gene/environment interactions that may increase cancer risk in human populations when β-pol is insufficient. In the β-pol heterozygous mouse model, we previously showed an increased sensitivity to carcinogens that induce damage similar to endogenous damage (1). This type of information is limited in its long-term implications because a wide spectrum of damages is induced by most carcinogens that may not mimic endogenous DNA damage. Although these studies will ultimately be necessary to determine whether BER deficiency increases susceptibility to environmental exposures, they do not adequately address the issue of sensitivity to endogenous damage. This is an important consideration, as this type of damage is not limited by exposure but is present constantly as byproducts of metabolism. To address the effects of endogenous stress, we have used aging to create an experimental condition in which endogenous damage accumulates at an accelerated rate. When combined with a reduced ability to process DNA damage, this may lead an increased rate of spontaneous tumor formation. The validity of our approach has been shown by van Remmen et al., who showed that accelerated accumulation of endogenous oxidative DNA damage resulted in a 100% increase in tumor risk (7).

Cancer rates increase exponentially with age, and it is possible that the age-related accumulation of spontaneous DNA damage is causally related to the cancer phenotype at old age. We suggest that loss of DNA repair capacity contributes to these events. We previously reported that with age, BER capacity and β-pol levels declined in all tissues examined by 50% on average (4). Mice heterozygous for β-pol already express 50% lower levels of β-pol transcripts, protein, and activity (1), in line with gene dosage. The combined reduction of β-pol level and BER capacity with age and

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β-pol heterozygosity could increase levels of DNA damage, potentially increasing cancer susceptibility. The present study characterizes the effect of life long reduction of β-pol on genomic integrity and aging.

Materials and Methods

Animals/life span. Experiments were done in male-specific pathogen-free mice in accordance with the NIH guidelines for the use and care of laboratory animals, and the animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio, the Subcommittee for Animal Studies at Audie L. Murphy Memorial Veterans Hospital, and the Wayne State University Animal Investigation Committee. Male mice were maintained under barrier conditions in microisolator cages on a 12-hour light/dark cycle and were fed a standard mouse lab chow and water *ad libitum*.

Mice heterozygous for the *DNA polymerase β* gene (β-pol$^{+/-}$) were created in Rajewsky's laboratory by deletion of the promoter and the first exon of the *β-pol* gene (8). Homozygous deletion of β-pol results in embryonic lethality, but the heterozygous mice survive and seem to be normal and are fertile; there is no retardation in food intake, weight gain, or growth rate. All mice were backcrossed into C57BL/6 for at least 18 generations. The genotype of the mice was determined by Southern blot analysis as described by Cabelof et al. (1). To determine the effect of β-pol haploinsufficiency on mutation frequency, the β-pol$^{+/-}$ mice were crossbred to *LacZ* transgenic mice (9). These cross-breedings produced mice heterozygous for the *β-pol* gene and hemizygous for *LacZ* transgene (*β-pol*$^{+/+}$/*LacZ* and *β-pol*$^{+/-}$/*LacZ*).

Life span. Longitudinal data were collected in β-pol$^{+/+}$ (n = 60) and β-pol$^{+/-}$ (n = 67) mice housed at the Barshop Institute for Longevity and Aging Studies at the University of Texas Health Science Center at San Antonio. After weaning, male β-pol$^{+/+}$ and β-pol$^{+/-}$ mice were housed four per cage and fed commercial mouse chow (Teklad Diet LM485) *ad libitum*. Age at natural death was recorded. Systematic macroscopic postmortem analysis of all mice was carried out.

Survival analysis was done on the life span data for the β-pol$^{+/+}$ (n = 60) and β-pol$^{+/-}$ (n = 67) mice according to standard demographic procedures plotting survival against time. Statistical difference between mortality rates was determined using the WinModest (10) program. WinModest analyzed the fit of our data to Gompertz, Gompertz-Makeham, and logistic models and determined the variables of the best fitting model. Maximum log likelihoods were used to determine the model that best describes our data, and the WinModest program calculated the associated probabilities using a χ^2 distribution.

Tumor cohort. Three cohorts of β-pol$^{+/+}$ (combined n = 25) and β-pol$^{+/-}$ (combined n = 32) mice were terminated at 24 to 26 months of age. A complete necropsy was done on all mice that died early or were sacrificed at termination. All animals were sacrificed by asphyxiation with carbon dioxide and necropsied within 5 minutes of death. At necropsy, all organs and tissues were examined for grossly visible lesions. All organs, including gross abnormalities, were fixed in 10% neutral buffered formalin, trimmed, embedded in paraffin, sectioned to a thickness of 5 to 6 μm, stained with H&E, and examined microscopically. Preparation of slides for histopathology evaluation was done by pathologists at the Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio (cohort 1) or the Laboratory of Experimental Pathology at the National Institutes of Environmental Health Sciences (cohorts 2 and 3).

RNA isolation and transcript determination. Total RNAs were isolated from the brain, spleen, and liver of young (4-6 months) β-pol$^{+/+}$ and old (24-26 months) β-pol$^{+/+}$ and β-pol$^{+/-}$ mice using the VersaGene RNA isolation system (Gentra, Minneapolis, MN) per manufacturer's protocol. cDNAs were synthesized from 2 μg RNA using random hexamer primers and a reverse transcription-PCR kit (Perkin-Elmer, Boston, MA) and purified with the QIAquick PCR Purification kit (Qiagen, Valencia, CA). β-Pol transcripts and 18S RNA transcripts were quantitated with a LightCycler real-time PCR machine (Roche, Indianapolis, IN). PCR reactions contained 2 μL purified cDNA, 4 mmol/L $MgCl_2$, 0.5 μmol/L each of sense and antisense primers, and 2 μL FastStart DNA Master SYBR Green I enzyme-SYBR reaction mix (Roche). Primer sequences: β-pol, sense 5′-CTGGAAAAGGGCTTCACAATCAATG-3′ and antisense 5′-GCGCCACTG-GATGTAATCAAAAATG-3′; 18S RNA, sense 5′-GTAACCCGTTGAACCC-CATT-3′ and antisense 5′-CCATCCAATCGGTAGTAGCG-3′. For all amplifications, PCR conditions consisted of an initial denaturing step of 99°C for 10 minutes followed by 35 to 55 cycles of 96°C for 10 seconds, 62°C for 10 seconds, and 72°C for 5 seconds, with a melting curve analysis from 40°C to 99°C to confirm specificity. External standards were prepared by amplification of cDNAs for β-pol and 18S RNA. The amplicons were cloned into pGEM-T Easy vector, linearized with *Apa*I, and used to prepare external standard curves. β-Pol transcripts were normalized to 18S RNA. Results are expressed as mean values from five animals per experimental group.

DNA isolation and mutation frequency determination. Genomic DNA was isolated from normal tissue by incubating tissue samples in lysis buffer [10 mmol/L Tris-HCl (pH 8), 10 mmol/L EDTA, 150 mmol/L NaCl, 1% SDS, 0.5 mg/mL proteinase K, 120 μg/mL RNase A] overnight at 50°C while rotating followed by extraction with phenol/chloroform/isoamyl alcohol and chloroform. Mutant frequencies were determined from 20 μg of genomic DNA digested with *Hin*dIII. Linearized plasmids were recovered using magnetic beads precoated with lacZ/lacI fusion protein. After washing, plasmid DNA was eluted using isopropyl-L-thio-B-D-galactopyranoside, circularized with T4 DNA ligase, and electroporated into *Escherichia coli* host cells. To determine the number of transformants recovered, 0.1% of the transformed bacterial cells were plated in agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. The remaining transformants were plated in 0.3% phenyl-galactoside to select for cells harboring plasmids with lacZ mutations. Mutant frequencies were calculated as the number of mutant colonies divided by the number of recovered transformants. Each mutant frequency determination point was based on at least 300,000 recovered plasmids per culture.

Metaphase spreads. Blood was obtained by cardiac puncture from old (24-26 months) male β-pol$^{+/+}$ (n = 6) and β-pol$^{+/-}$ (n = 6) mice. The blood was drawn into heparinized syringes and washed twice in RPMI 1640. Whole blood (1 mL) was added to 9 mL RPMI 1640 (with L-glutamine) containing 12.5% fetal bovine serum, 1% penicillin/streptomycin, 1% sodium heparin, and 10 mg/mL lipopolysaccharide. The cultures were incubated at 37°C under 5% CO_2 for 36 hours. Colcemid (0.2 μg/mL) was added to the cultures at 24 hours. Cells were harvested by swelling in 75 mmol/L KCl for 30 minutes, fixed thrice in 3:1 (v/v) methanol/glacial acetic acid, and dropped onto humidified slides. Slides were Giemsa stained. Blinded to genotype, 200 metaphase cells from each animal were scored for numerical chromosomal aberrations. Overlapping cells or cells that were not clearly identifiable as individual cells were excluded from analysis. Data are expressed as the percentage of hypoploid cells (total percentage of cells with <40 chromosomes).

Protein isolation and Western analysis. Protein was obtained using the CelLytic NuclearExtraction kit (Sigma, St. Louis, MO) per manufacturer's protocol. Nuclear extracts from liver tissues of 10 β-pol$^{+/+}$ and 10 β-pol$^{+/-}$ animals were subjected to SDS-PAGE and transferred to nitrocellulose using a Bio-Rad semidry transfer apparatus according to the manufacturer's protocol. Equal loading and transfer of proteins was determined by reversible staining of the membrane with MemCode (Pierce, Rockford, IL). Western analysis was accomplished using affinity-purified monoclonal antisera developed against mouse p53 (pAb240). Bands were detected by ChemiImager after incubation in SuperSignal Chemiluminescent Substrate luminol/enhancer and SuperSignal chemiluminescent Substrate stable peroxide solution (Pierce). Intensity of the bands was quantified by ChemiImager, and the data are expressed as the integrated density of the band per μg protein loaded.

Statistical analysis. Statistical significance between means between *β-pol*$^{+/+}$ and *β-pol*$^{+/-}$ mice were analyzed by two-factor ANOVA followed by Sidak's multiple comparison test. Statistical significance of tumor incidence between *β-pol*$^{+/+}$ and *β-pol*$^{+/-}$ mice, and odds ratios (OR) were

calculated as described by Dowdy et al. (11). $P < 0.05$ was considered statistically significant. For statistical analysis of mortality curves, associated probabilities were calculated by χ^2 distribution using the WinModest program (10).

Results

We previously characterized β-pol heterozygous mice and found that young mice did not develop spontaneous tumors. However, young mice did show increased mutagenicity in response to the DNA-damaging agent dimethyl sulfate, suggesting that 50% loss of β-pol might increase cancer susceptibility. In this study, we have evaluated the effect of aging in this mouse model and find that aging and heterozygous loss of β-pol interact to increase cancer risk. Pathology was collected for cohorts of 24- to 26-month-old β-pol$^{+/+}$ and β-pol$^{+/-}$ mice, and a significant increase in the incidence of spontaneous lymphomas was found in the β-pol haploinsufficient mice (Fig. 1). Only 4% of old β-pol$^{+/+}$ mice (1 of 25) developed lymphoma, whereas 22% of 24- to 26-month-old β-pol$^{+/-}$ mice (7 of 32) developed lymphoma ($P < 0.01$). The 4% incidence observed in 24- to 26-month-old male C57BL mice is consistent with data from Smith et al. (12), in which life span and cancer incidence were evaluated in several long-lived inbred mouse strains, including C57BL. Thus, β-pol$^{+/-}$ mice are nearly seven times more likely to develop lymphoma than are their wild-type counterparts (OR, 6.7 $\pm$ 0.77; $P < 0.007$).

In comparing the lymphomas observed in the aged β-pol$^{+/-}$ mice to the lymphoma observed in one aged β-pol$^{+/+}$ mouse, those in the β-pol$^{+/-}$ mice had involvement in multiple lymphoid organs, a phenotype absent in the β-pol$^{+/+}$ mouse. We also analyzed nonneoplastic pathologies in the 24- to 26-month-old cohort mice ($n = 23$). Lymphocyte infiltration was more severe in the β-pol$^{+/-}$ mice than the β-pol$^{+/+}$ mice, and 37.5% of β-pol$^{+/-}$ mice exhibited lymphoid hyperplasia, whereas none of the β-pol$^{+/+}$ mice exhibited this phenotype. These data establish an abnormal lymphoproliferation in the β-pol$^{+/-}$ mice and provide supporting evidence for the observation that β-pol heterozygosity increases the incidence of lymphoma.

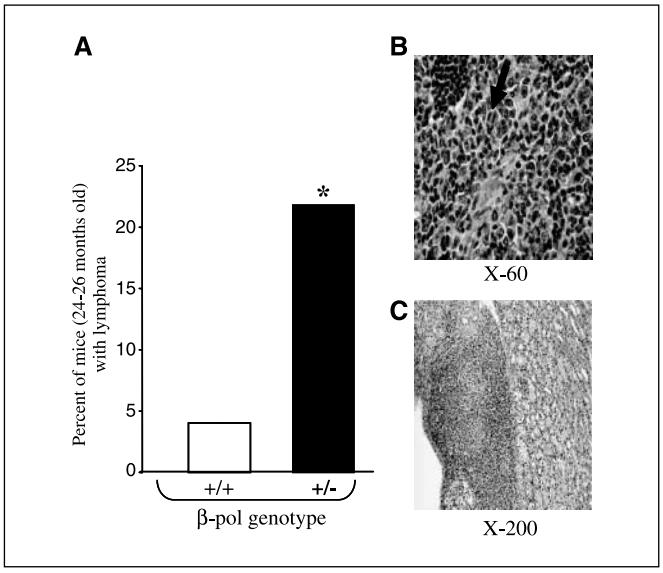

Figure 1. Development of lymphoma in β-pol$^{+/+}$ and β-pol$^{+/-}$ mice. *A,* complete necropsies as described in Materials and Methods were completed on β-pol$^{+/+}$ and β-pol$^{+/-}$ mice terminated at 24 to 26 months of age. *, $P < 0.05$, significantly different from control. *B,* photographic image of mesenteric lymph node displaying lymphoma in β-pol$^{+/-}$ mouse. *Arrow,* neoplastic cell in mitosis. *C,* photographic image of kidney infiltrated by lymphoma cells. □, β-pol$^{+/+}$; ■, β-pol$^{+/-}$.

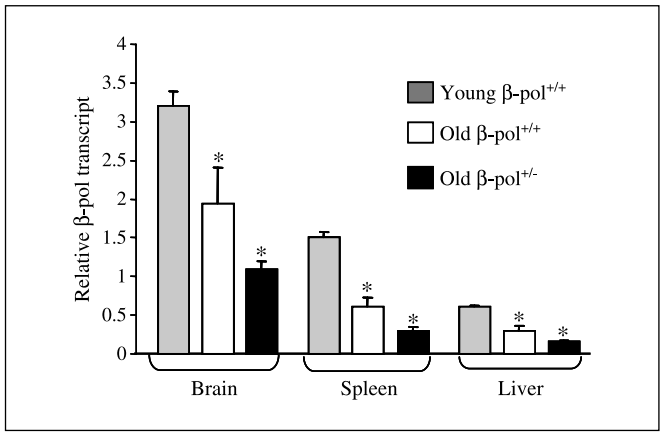

Figure 2. Maintenance of gene-dosage effect of β-pol heterozygosity on transcript level with age. cDNAs were prepared from RNA isolated from brain, spleen, and liver tissue of young β-pol$^{+/+}$, old β-pol$^{+/+}$, and old β-pol$^{+/-}$ mice. β-Pol transcript levels were determined by real-time PCR analysis and normalized to 18S RNA. *Columns,* mean from five animals in each group; *bars,* SE. *, $P < 0.05$, significantly different from control.

In addition to the cohort study, we also did a systematic evaluation of end-of-life pathology in the animals used in the life span study. We collected pathology data for all tumors occurring at a frequency of >5% (data not shown). We observed a significant increase in the number of adenocarcinomas in the β-pol$^{+/-}$ mice such that the β-pol$^{+/-}$ mice were 2.7 times more likely to develop adenocarcinoma ($P < 0.05$) than their wild-type counterparts. Additionally, 19.7% of the β-pol$^{+/-}$ animals died bearing multiple tumors compared with only 5% of the β-pol$^{+/+}$ animals ($P < 0.05$). This increase in tumor multiplicity resulted in an average of 0.97 tumors per mouse for β-pol$^{+/-}$ mice compared with 0.80 tumors per mouse for the β-pol$^{+/+}$ mice. In contrast to the significant increase in the incidence of lymphoma observed in the 24- to 26-month-old mice, there was no significant increase in the number of lymphomas at the end of life between genotypes (55% incidence, β-pol$^{+/+}$; 61% incidence, β-pol$^{+/-}$). As such, this is a model in which we see both an acceleration of normal, age-related tumors (lymphomas) developing alongside an increased susceptibility to epithelial tumors (adenocarcinomas), which do not typically occur at a high incidence in C57BL/6 mice.

To establish a role for β-pol in the development of these tumors, we needed to show that the β-pol heterozygous state is maintained with age in the β-pol$^{+/-}$ mice. We analyzed β-pol transcript level in brain, spleen, and liver tissue of young (4-6 months) β-pol$^{+/+}$ and old (24-26 months) β-pol$^{+/+}$ and β-pol$^{+/-}$ mice. We found that the 50% reduction in β-pol transcripts observed in response to β-pol heterozygosity in young animals was maintained in aged animals (Fig. 2). These data allow us to rule out the possibility that adaptation to β-pol heterozygosity could occur over the life of the animal, perhaps through altered expression of the wild-type allele. These data also confirm previously reported age-related loss of β-pol and tissue-specific differences in β-pol expression (13). By Western and *in vitro* BER analysis, we evaluated β-pol protein levels and BER capacity in liver protein extracts from old β-pol$^{+/+}$ and β-pol$^{+/-}$ mice (data not shown) and likewise found maintenance of the heterozygous state at the level of protein and enzymatic activity.

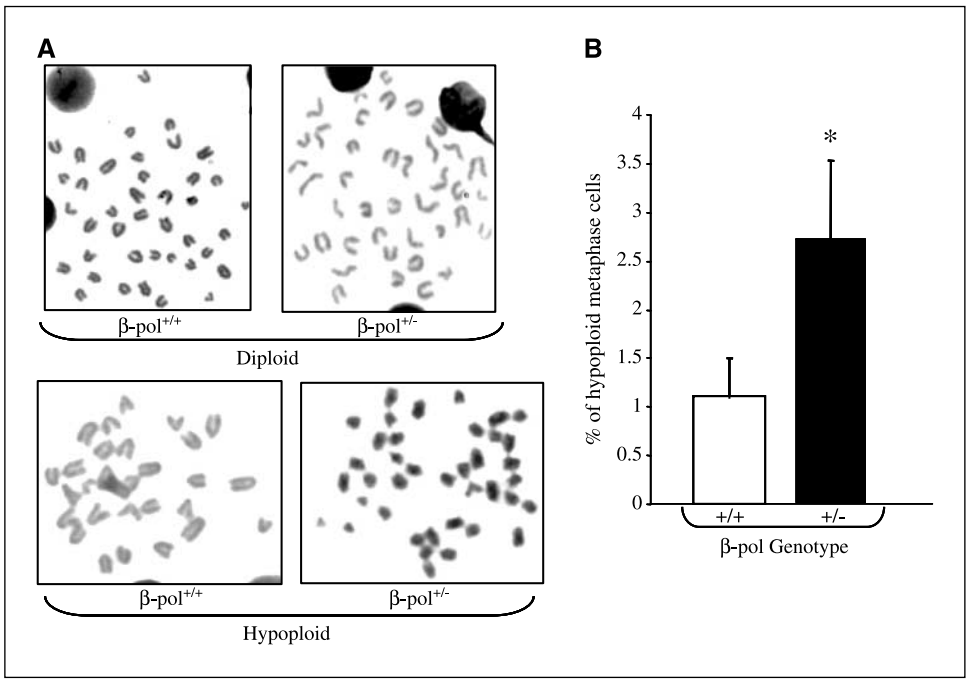

Figure 3. Lifelong reduction in β-pol increases chromosomal instability. Metaphase cells were prepared from peripheral blood of old (24-26 months) β-pol$^{+/+}$ and β-pol$^{+/-}$ mice as described in Materials and Methods. *A,* representative metaphase images. *B,* percentage of all metaphase cells with <40 chromosomes. *Columns,* average for data obtained from six animals in each group; *bars,* SE. *, $P < 0.05$, significantly different from control.

To determine whether the very low levels of β-pol observed in brain, spleen, and liver of β-pol$^{+/-}$ mice resulted in DNA sequence alterations, we measured mutation frequencies at the *lacZ* transgene locus in old β-pol$^{+/+}$ and β-pol$^{+/-}$ mice in the brain, spleen, and liver. We observed no effect of β-pol heterozygosity on the accumulation of *lacZ* mutants in any tissue (data not shown). Presenting the data as the average ± SD mutant frequency ($\times 10^{-5}$) across genotypes, we find a mutant frequency of 9.5 ± 1.4 in old liver, 7.7 ± 2.6 in old brain, and 6.9 ± 1.3 in old spleen. This is consistent with previous determinations of *lacZ* mutant frequency in these tissues from old mice (14, 15).

To evaluate whether β-pol heterozygosity alters chromosomal stability, we evaluated the effect of aging on first division numerical chromosome changes in old β-pol$^{+/+}$ and β-pol$^{+/-}$ mice. This study was a logical follow-up to the initial characterization of these mice, in which we showed that younger β-pol$^{+/-}$ mice displayed a 10-fold increase in the level of premature centromere separation (1). In the present study, we observed that β-pol heterozygosity resulted in a 2.5-fold increase in the number of hypoploid cells (Fig. 3; $P < 0.05$). This shows that loss of β-pol results in chromosomal instability, consistent with the previous finding that loss of β-pol induces premature centromere separation.

Loss of p53 plays an obvious role in the development of tumors. We analyzed both nuclear and cytoplasmic p53 protein levels in livers of old β-pol$^{+/+}$ and β-pol$^{+/-}$ mice to determine whether there might be an effect of β-pol loss on subcellular localization of p53, as a persistence of DNA damage might result in enhanced nuclear accumulation. We found no effect of β-pol loss on accumulation of p53 protein in either the nucleus or cytoplasm (Fig. 4).

Finally, we undertook a detailed characterization of the life span using a large number of β-pol$^{+/+}$ and β-pol$^{+/-}$ mice. In Fig. 5, we show that the mean and maximum survival was similar for the two genotypes. However, it is evident from the survival curves that the β-pol$^{+/+}$ and β-pol$^{+/-}$ curves are not identical. It is interesting that the βpol$^{+/-}$ mice seemed to have a survival advantage early in life, as 10% of the β-pol$^{+/+}$ mice died before any of the β-pol$^{+/-}$ mice had died. Likewise, 10% survival at the end of life seems to be greater in the β-pol$^{+/+}$ mice than in the β-pol$^{+/-}$ mice. The net effect is that the slope of the survival curve for the β-pol$^{+/-}$ mice is steeper than that for the wild-type mice, suggesting that once these mice start to die they do so at a faster rate.

To further address this, we have analyzed mortality rates of the β-pol$^{+/-}$ mice and the wild-type littermates using Gompertz variables and a maximum likelihood estimation approach as described by Pletcher et al. (10). In spite of the similarities in mean and maximum life span between genotypes, a separate model fitting showed that age-specific mortality differs between the genotypes, confirming the apparent differences observed in Fig. 5. This analysis establishes a slight but significant acceleration in the age-dependent mortality rate ($R^2 = 0.813$, β-pol$^{+/+}$; $R^2 = 0.945$, β-pol$^{+/-}$; $P < 0.05$; Fig. 6). It is noteworthy that the hazard curve

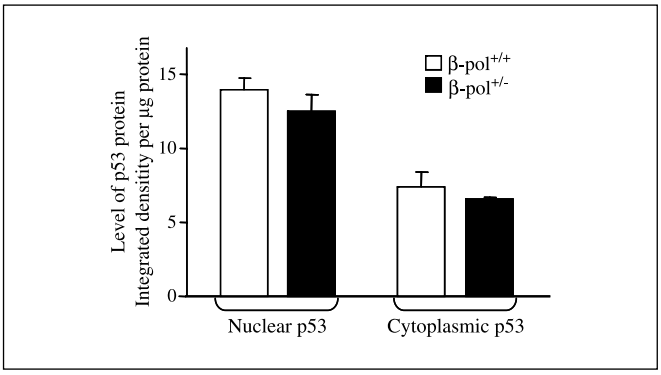

Figure 4. β-pol heterozygosity does not alter p53 protein expression level with advanced age. *A,* level of nuclear p53 protein in liver of old β-pol$^{+/+}$ and old β-pol$^{+/-}$ mice was determined by Western blot analysis as described in Materials and Methods. *B,* level of cytoplasmic p53 protein in liver of old β-pol$^{+/+}$ and old β-pol$^{+/-}$ mice was determined by Western blot analysis as described in Materials and Methods. *Columns,* average for data obtained from at least 10 animals in each group; *bars,* SE.

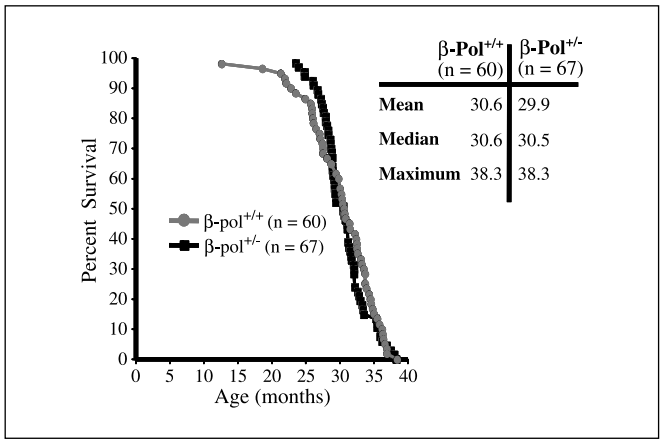

Figure 5. Life span and survival of β-pol$^{+/+}$ and β-pol$^{+/-}$ mice. Mean, median, and maximum survival data were determined in β-pol$^{+/+}$ (n = 60) and β-pol$^{+/-}$ (n = 67) mice by plotting the age of natural death as described in Materials and Methods.

for the β-pol$^{+/-}$ mice shifted from protective to detrimental between 25 and 30 months of age.

Discussion

The risk for developing lymphoma and adenocarcinoma is significantly increased by β-pol heterozygosity. Loss of other BER genes increases cancer risk as well. Heterozygous loss of APE is associated with a 12% incidence of lymphoma and a 25% incidence of total tumors (16). Homozygous loss of Uracil DNA Glycosylase (UNG$^{-/-}$) has little effect in young mice (17) but with age results in an approximate 10-fold increased risk for lymphoma (18). The 7-fold increase in lymphoma incidence observed in the β-pol$^{+/-}$ mice had no effect on mean life span, likely reflecting tumors with slow growth properties and/or a long latency. Slow-growing tumors in old mice would likewise be consistent with observations that mortality from cancer declines with age (19, 20). Because the C57BL strain is lymphoma prone (12), we suggest that our observations reflect an accelerated onset of lymphoma in the β-pol$^{+/-}$ mice. The 22% lymphoma incidence observed in the β-pol$^{+/-}$ mice at 24 to 26 months is not seen in wild-type mice until ~30 months, and the average age of onset for wild-type mice is 27 months (12). In light of only a small difference in the incidence of lymphoma at the end of life (55%, β-pol$^{+/+}$ compared with 61%, β-pol$^{+/-}$), our findings support an acceleration in development of age-related tumors in response to β-pol haploinsufficiency. It may be remarkable that dietary restriction suppresses lymphomagenesis (21), specifically thymic lymphoma (22). As loss of β-pol clearly accelerates onset of lymphoma, perhaps up-regulation of nuclear DNA repair capacity and β-pol expression by dietary restriction (13, 23) contrarily inhibits onset of lymphoma. β-Pol also plays a role in development of solid tumors as we observed an increased incidence of adenocarcinomas that are tumors to which the C57BL strain is not predisposed, supporting a tumor suppressor role for β-pol. This is further supported by our observations of increased tumor multiplicity and tumor-bearing load in response to β-pol haploinsufficiency.

The precise mechanism by which these tumors develop is not obvious and is likely the result of several factors. β-Pol levels may need to fall below a critical threshold level in order for tumors to develop. As we show here, the combination of aging and β-pol heterozygosity has a cumulative effect on β-pol expression. That is, old β-pol$^{+/-}$ mice express very low levels of β-pol as a result of a combination of the gene-targeted decline in expression and the age-related decline in expression. Additionally, β-pol loss alone may not be sufficient for tumor development. Perhaps, a combination of β-pol loss and age-related accumulation of DNA damage is required for tumor development. Another contributing factor may relate to loss of a p53 response with age. Young β-pol heterozygotes accumulate p53 (40% increase; ref. 1), but the old heterozygous mice do not (Fig. 4). We suggest that in young animals, an accumulation of DNA damage resulting from β-pol loss results in p53 accumulation, but that in old animals, this response is lost. In support, loss of the p53 DNA damage response has been shown to occur with age (24, 25).

Tumors develop in spite of an inability to detect DNA sequence mutations. We propose a mechanism by which a reduced ability to process endogenous damage results in accumulating DNA strand breaks that drive chromosomal instability and ultimately lead to tumors. Exposure to oxidative stress in β-pol$^{+/-}$ mice leads to an accumulation of DNA repair intermediates (26) that may drive recombinational processes. Indeed, Fotiadou et al. (27) and Sobol et al. (28) have shown an effect of β-pol loss on chromosomal instability. This supports our initial finding in 12-month-old β-pol$^{+/-}$ mice of a 10-fold increase in the level of premature centromere separation in bone marrow metaphase cells (1). Our present observation that the percentage of hypoploid cells increases in the β-pol$^{+/-}$ mice is consistent with malsegregation of chromosomes that could lead to hypoploidy. A role for β-pol in chromosomal segregation separate from its role in BER is, perhaps, suggested by the finding that β-pol decorates the microtubles, (29).

We were surprised that the increase in tumor incidence in the β-pol$^{+/-}$ mice did not alter either mean or maximum life span. Van Remmen et al. also showed increased tumor incidence without changes in life span in Sod2$^{+/-}$ mice (7). However, the shape of the survival curves for the Sod2$^{+/-}$ mice and Sod2$^{+/+}$ mice were identical, whereas those for the β-pol$^{+/-}$ and β-pol$^{+/-}$ mice were not.

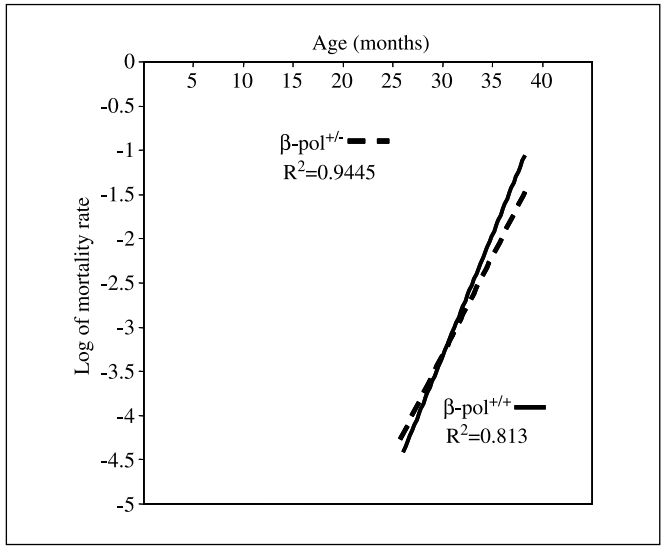

Figure 6. Mortality curves of β-pol$^{+/+}$ and old β-pol$^{+/-}$ mice. Using the WinModest program as described in Materials and Methods, we determined the mortality rates for each data set as described in Materials and Methods. Maximum log likelihoods were used to determine the model that best describes our data, and the WinModest program calculated the associated probabilities. *, P < 0.05, significantly different from control.

We have identified a subtle but significant effect of β-pol heterozygosity on mortality rate, supporting a role for haploinsufficiency of β-pol on aging. Nearly all human diseases of segmental premature aging and mouse models designed to mimic these diseases are associated with DNA repair deficiency, supporting the argument that aging results from a gradual accumulation of DNA damage (30). Some of these DNA repair mutants exhibit extreme effects on life span (31–33). Other mutants exhibit less extreme but very significant effects on survival (32, 34, 35). The homozygous UNG mutant, the only BER mutant other than the β-pol mutant on which life span data has been collected, shows a reduction in mean life span (18). The small magnitude of effect on mortality in the β-pol mutant most likely relates to the heterozygous status of the β-pol$^{+/-}$ mouse, in fact, making the point that haploinsufficiency can affect aging. One example is seen in Down syndrome, a condition characterized by moderate premature aging. Here, β-pol expression is reduced to haploinsufficient levels (36), which we have verified in Down syndrome cell lines as well.[8]

In summary, the major finding of this work is establishment of the phenotypic importance of the inability to process endogenous damage. Loss of β-pol both increases tumor development and alters survival. This effect of β-pol on mortality rate is quite distinct from that observed in other DNA repair deficiencies. The finding that loss of β-pol provides a protective effect early in life, which is lost by 28 months of age, suggests an exciting possibility that an adaptive response occurs early but is lost late in life. It is possible that alternative DNA repair pathways accommodate for the loss in BER in young animals. Loss of this accommodation with age would be consistent with observations that DNA repair capacity decreases with age. Our data suggest a role for p53 as a mediator of this adaptive response, as the p53 DNA damage response is intact in the young mice and absent in the old mice and is consistent with the increased development of lymphoma. The mechanism by which this response is lost with age is presently unclear. Developing an understanding may shed light on normal mechanisms of aging and on the mechanisms behind development of age-related cancers.

[8] Unpublished data.

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References

1. Cabelof DC, Guo Z, Raffoul JJ, et al. Base excision repair deficiency caused by polymerase beta haploinsufficiency: accelerated DNA damage and increased mutational response to carcinogens. Cancer Res 2003;63:5799–807.
2. Vijg J. Somatic mutations and aging: a re-evaluation. Mutat Res 2000;447:117–35.
3. Hasty P, Campisi J, Hoeijmakers J, van Steeg H, Vijg J. Aging and genome maintenance: lessons from the mouse? Science 2003;299:1355–9.
4. Cabelof DC, Raffoul JJ, Yanamadala S, Ganir C, Guo Z, Heydari AR. Attenuation of DNA polymerase beta-dependent base excision repair and increased DMS-induced mutagenicity in aged mice. Mutat Res 2002;500:135–45.
5. Intano GW, Cho EJ, McMahan CA, Walter CA. Age-related base excision repair activity in mouse brain and liver nuclear extracts. J Gerontol A Biol Sci Med Sci 2003;58:205–11.
6. Hoeijmakers J. Genome maintenance mechanisms for preventing cancer. Nature 2001;411:366–74.
7. van Remmen H, Ikeno Y, Hamilton M, et al. Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. Physiol Genomics 2003;16:29–37.
8. Gu H, Marth JD, Orban PC, Mossmann H, Rajewsky K. Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. Science 1994;265:103–6.
9. Boerrigter M, Dolle ME, Martus HJ, Gossen JA, Vijg J. Plasmid-based transgenic mouse model for studying *in vivo* mutations. Nature 1995;377:657–9.
10. Pletcher SD, Khazaeli AA, Curtsinger JW. Why do life spans differ? Partitioning mean longevity differences in terms of age-specific mortality parameters. J Gerontol A Biol Sci Med Sci 2000;55:B381–9.
11. Dowdy S, Weardon S, Chilko D, editors. Statistics for research. 3rd ed. Hoboken (NJ): John Wiley and Sons, Inc.; p. 118–20 and 168–71.
12. Smith GS, Walford RL, Mickey MR. Lifespan and incidence of cancer and other diseases in selected long-lived inbred mice and their F 1 hybrids. J Natl Cancer Inst 1973;50:1195–213.
13. Cabelof DC, Yanamadala S, Raffoul JJ, Guo Z, Soofi A, Heydari AR. Caloric restriction promotes genomic stability by induction of base excision repair and reversal of its age-related decline. DNA Repair (Amst) 2003;2:295–307.
14. Dollé MR, Giese H, Hopkins CL, Martus HJ, Hausdorff JM, Vijg J. Rapid accumulation of genome rearrangements in liver but not in brain of old mice. Nat Genet 1997;17:431–4.
15. Ono T, Ikehata H, Nakamura S, et al. Age-associated increase of spontaneous mutant frequency and molecular nature of mutation in newborn and old lacZ-transgenic mouse. Mutat Res 2000;447:167–77.
16. Meira LB, Devaraj S, Kisby GE, et al. Heterozygosity for the mouse Apex gene results in phenotypes associated with oxidative stress. Cancer Res 2001;61:5551–7.
17. Nilsen H, Rosewell I, Robins P, et al. Uracil-DNA glycosylase (UNG)-deficient mice reveal a primary role of the enzyme during DNA replication. Mol Cell 2000;5:1059–65.
18. Nilsen H, Stamp G, Andersen S, et al. Gene-targeted mice lacking the Ung uracil-DNA glycosylase develop B-cell lymphomas. Oncogene 2003;22:5381–6.
19. Pompei F, Polkanov M, Wilson R. Age distribution of cancer in mice: the incidence turnover at old age. Toxicol Ind Health 2001;17:7–16.
20. Lipman RD, Smith DE, Blumberg JB, Bronson RT. Effects of caloric restriction or augmentation in adult rats: longevity and lesion biomarkers of aging. Aging (Milano) 1998;10:463–70.
21. Weindruch R, Walford RL. Dietary restriction in mice beginning at 1 year of age: effect on life-span and spontaneous cancer incidence. Science 1982;215:1415–8.
22. Hursting SD, Perkins SN, Phang JM. Calorie restriction delays spontaneous tumorigenesis in p53-knockout transgenic mice. Proc Natl Acad Sci U S A 1994;91:7036–40.
23. Stuart JA, Karahalil B, Hogue BA, Souza-Pinto NC, Bohr VA. Mitochondrial and nuclear DNA base excision repair are affected differently by caloric restriction. FASEB J 2004;18:595–7.
24. Cabelof DC, Raffoul JJ, Ge Y, Van Remmen H, Matherly LH, Heydari AR. Age-related loss of the DNA repair response following exposure to oxidative stress. J Gerontol A Biol Sci Med Sci 2006;61:427–34.
25. Goukassian D, Gad F, Yaar M, Eller MS, Nehal US, Gilchrest BA. Mechanisms and implications of the age-associated decrease in DNA repair capacity. FASEB J 2000;14:1325–34.
26. Cabelof DC, Raffoul JJ, Yanamadala S, Guo Z, Heydari AR. Induction of DNA polymerase beta-dependent base excision repair in response to oxidative stress *in vivo*. Carcinogenesis 2002;23:1419–25.
27. Fotiadou P, Henegariu O, Sweasy JB. DNA polymerase beta interacts with TRF2 and induces telomere dysfunction in a murine mammary cell line. Cancer Res 2004;64:3830–7.
28. Sobol RW, Kartalou M, Almeida KH, et al. Base excision repair intermediates induce p53-independent cytotoxic and genotoxic responses. J Biol Chem 2003;278:39951–9.
29. Conlon KA, Miller H, Rosenquist TA, Zharkov DO, Berrios M. The murine DNA glycosylase NEIL2 (mNEIL2) and human DNA polymerase beta bind microtubules *in situ* and *in vitro*. DNA Repair (Amst) 2005;4:419–31.
30. Kirkwood TB. Evolution of ageing. Nature 1977;270:301–4.
31. Weeda G, Donker I, de Wit J, et al. Disruption of mouse ERCC1 results in a novel repair syndrome with growth failure, nuclear abnormalities and senescence. Curr Biol 1997;7:427–39.
32. de Boer J, Andressoo JO, de Wit J, et al. Premature aging in mice deficient in DNA repair and transcription. Science 2002;296:1276–9.
33. Murai M, Enokido Y, Inamura N, et al. Early postnatal ataxia and abnormal cerebellar development in mice lacking Xeroderma pigmentosum Group A and Cockayne syndrome Group B DNA repair genes. Proc Natl Acad Sci U S A 2001;98:13379–84.
34. Vogel H, Lim DS, Karsenty G, Finegold M, Hasty P. Deletion of Ku86 causes early onset of senescence in mice. Proc Natl Acad Sci U S A 1999;96:10770–5.
35. Espejel S, Martin M, Klatt P, Martin-Caballero J, Flores JM, Blasco MA. Shorter telomeres, accelerated ageing and increased lymphoma in DNA-PKcs-deficient mice. EMBO Rep 2004;5:503–9.
36. Raji NS, Rao KS. Trisomy 21 and accelerated aging: DNA-repair parameters in peripheral lymphocytes of Down's syndrome patients. Mech Ageing Dev 1998;100:85–101.