Disruption of the NAD(P)H:Quinone Oxidoreductase 1 (NQO1) Gene in Mice Causes Myelogenous Hyperplasia

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ABSTRACT

NAD(P)H:quinone oxidoreductase1 (NQO1) is a cytosolic protein that reduces and detoxifies quinones and their derivatives, thus protecting cells against redox cycling and oxidative stress. Disruption of the NQO1 gene in mice caused myeloid hyperplasia of bone marrow and highly significant increases in blood neutrophils, eosinophils, and basophils. NQO1-null mice also showed a decrease in lymphocytes and WBCs as compared with wild-type mice. Various techniques also demonstrated an increase in megakaryocytes without an increase in blood platelets. Histological analysis of liver, kidney, spleen, and thymus did not demonstrate a difference between wild-type and NQO1-null mice or a sign of infection. Blood cultures and urine analysis also did not demonstrate any sign of infection in NQO1-null and wild-type mice. Additional analysis of the bone marrow from NQO1-null mice revealed that loss of NQO1 alters the intracellular redox status because of accumulation of NADP(H), cofactors for NQO1. This causes a reduction in the levels of pyridine nucleotides and tumor suppressor proteins p53 and p73, and a decrease in apoptosis. The decrease in apoptosis causes myelogenous hyperplasia in NQO1-null mice. These results demonstrate that NQO1 acts as an endogenous factor in the protection against myelogenous hyperplasia. This is significant because 2–4% of human individuals without known abnormalities, and >25% of individuals with benzene poisoning and acute myelogenous leukemia are homozygous for a mutant allele (P187S) of NQO1 and lack NQO1 protein/activity.

INTRODUCTION

NQO1 activity is ubiquitously present in all tissue types (1–3). NQO1 gene expression is induced in response to xenobiotics, anti-oxidants, oxidants, heavy metals, UV light, and ionizing radiation (1–4). Interestingly, NQO1 is part of an electrophilic and/or oxidative stress-induced cellular defense mechanism that includes the induction of more than two dozen genes (1–4). The other genes that are coordinately induced with the NQO1 gene include glutathione S-transferases, which conjugate hydrophobic electrophiles and reactive oxygen species to glutathione; UDP-glucuronosyltransferases, which catalyze the conjugation of glucuronic acid with xenobiotics and drugs for their excretion; epoxide hydrolase, which hydrolyzes epoxides; and γ-glutamylcysteine synthetase, which plays a key role in the regulation of glutathione metabolism (1–4). Therefore, the coordinated induction of these genes, including NQO1, provides the necessary cellular protection against free radical damage and oxidative stress.

Materials and Methods

Generation and Analysis of NQO1-null Mice. A detailed description of the generation of NQO1-null (NQO1−/−) mice is described (9). Briefly, the 5’ and 3’ homologous genomic sequences were 3.4 kb and 1.4 kb long, respectively, that flanked exon 6 of the NQO1 gene. In the targeting vector, a 2.0-kb Bam HI fragment containing exon 6 of the NQO1 gene was replaced with 2.0 kb of neocassette. This replacement was engineered to delete the carboxyl 101 amino acids of the NQO1 enzyme. This design effectively disrupted the NQO1 gene function. The homologous recombination-positive ES cells of 129Sv origin were used to generate chimeric mice (129Sv/C57BL6) and germ-line transmission was detected. Heterozygous mice from the F1 generation were normal and were interbred to generate homozygous NQO1-null mice. The chimeric mice were backcrossed with C57BL6 mice to generate C57BL6 NQO1-null mice used in the present studies. The NQO1-null mice were found to be normal in appearance and showed no discernible difference in their development or in their behavior compared with their wild-type NQO1+/− litter-mates (9). This was true for both male and female mice. In addition, the NQO1-null mice appeared to have normal reproductive capacity when compared with wild-type mice.

Peripheral Blood Analysis. Eleventh generation of NQO1-null and wild-type mice were anesthetized with isoflurane (Vedco, St. Joseph, MO) followed by i.p. injections of a combination anesthetic (2.14 mg ketamine, 0.43 mg xylazine, and 70 μg acepromazine). The animals were opened and 0.25 cc of blood was withdrawn from the heart with a 22-gauge needle. The syringes were coated with 0.5 m EDTA to prevent coagulation. The blood was immediately placed into EDTA-coated tubes (Microtainer; Fisher Scientific, Houston, TX) and thoroughly mixed. The blood samples were analyzed by a Technicon H1 analyzer.

Bone Marrow NQO1 Protein and Activity. The wild-type and NQO1-null mice from eleventh generation were sacrificed and their femurs removed. The bones were cut, and marrow flushed out and homogenized in a buffer containing 250 mM sucrose, 1 mM EDTA, and cytosol prepared by standard techniques (9). The cytosolic proteins (75 μg) were run on 10% SDS-PAGE, transferred to ECL nitrocellulose membranes and probed with polyclonal

Received 10/16/01; accepted 4/2/02.

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Supported by NIH Grant RO1 ES07943.

2 Two authors contributed equally to this work.

1 Supported by NIH Grant RO1 ES07943.

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3 The abbreviations used are: NQO1, NADPH:quinone oxidoreductase 1 or DT diaphorase; ECL, enhanced chemiluminescence; HPLC, high-performance liquid chromatography; PCNA, proliferating cell nuclear antigen; CEBP, CAAT/enhancer binding protein; AML, acute myelogenous leukemia; ES, embryonic stem; KOH, potassium hydroxide; KCN, potassium cyanide.
antibodies against NQO1. The cytosolic fractions were also analyzed for NQO1 activity by procedures described previously (9).

Analysis of Bone Marrow Cytospins. NQO1-null and wild-type mice were euthanized. The femurs were surgically removed from the animals. The heads of each femur were removed, and the bone was then flushed with ~1 ml of PBS containing 1 mM EDTA. The volume flushed from each bone was increased to 3 ml with PBS. The solution was mixed, and 150 μl were used for each cytospin preparation. The samples were spun onto slides using a Cyto-Tek Centrifuge (Sakura Scientific, Torrance, CA) at 1600 rpm for 10 min. Differential staining was done on the cytospin preparations using the HEMA 3 stain set from Fisher Scientific (Houston, TX). Additional slides containing bone marrow cells were fixed in 95% ethanol and stained with an antihuman myeloperoxidase antibody (DAKO, Glostrup, Denmark). The secondary antibodies and the development kit were obtained from Vector Laboratories (Burlingame, CA). The slides were stained according to the manufacturer’s protocol.

Histology. NQO1-null and wild-type mice were euthanized, and the femurs/sternums were surgically removed. The bones were placed in 10% neutral buffered formalin (Fischer Scientific, Houston, TX) for 24 h. After this time period, the bones were decalcified in TDB-2 Decalcifier (23% formic acid +9% sodium citrate; Shandon Lipsch, Pittsburgh, PA) for 24 h. The tissues were then embedded in paraffin and cut into 4-μm sections. The sections were placed onto slides and stained with H&E (Richard-Allan Scientific, Kalamazoo, MI). In addition to bone marrow, histological analysis was also performed on the liver, kidney, spleen, and thymus of wild-type and NQO1-null mice. These tissues were fixed in formalin, embedded in paraffin, cut into sections, and stained with H&E by standard procedures.

Cytogenetic Preparations. The wild-type and NQO1−/− male and female mice were injected i.p. with 0.2 ml of Colcemid (2.0 mg/ml stock solution) 1 h before killing. Bone marrow was aspirated in a hypotonic solution (0.075 M KCl) with the help of a syringe fitted with a 25-gauge needle. Cell clumps were broken into single-cell suspension by mild vortexing. Cells were suspended in KCl solution for 15–20 min at room temperature, fixed in acetic acid:methanol (1:3 by volume) and finally dropped onto glass slides for air-dried preparations. G banding was performed after standard laboratory procedures (10). Banded chromosomes were classified following the standard nomenclature of the Committee on Standardized Genetic Nomenclature for Mice (11). An average of 15–20 G banded metaphases was photographed and the complete karyotypes prepared from each animal using a Genetiscan (Perceptive System, Inc., Houston, TX). An additional 10–15 conventionally Giemsa-stained metaphase spreads from each animal were evaluated for any chromatin or chromosome-type aberrations and for the determination of model chromosome number.

Menadione Treatment. Menadione (Sigma Chemical Company, St. Louis, MO) was dissolved in DMSO. Menadione (5 mg/kg body weight) was administered intraperitoneally once every day for 3 days to wild-type and NQO1-null mice. Control mice received DMSO alone. The blood was drawn 24 h after the last injection and analyzed. In related experiments, the mice were euthanized, and bone marrow was collected and analyzed by procedures as described above.

NAD(P)H:NAD(P) Ratio in Bone Marrow. The following procedure was used to collect and process the tissue for determination of NAD(P) and NAD(P)H because of sensitivity of these molecules. The bone marrow was surgically removed while the mice were under anesthesia to avoid changes in the levels of the pyridine nucleotides. The bone marrow was then instantly placed in liquid nitrogen (12). While frozen, the ends of the bone were cut using a surgical blade, and while the marrow was thawing, it was flushed by using the solution containing 200 mM KCN, 1 mM bathophenanthroline, and 60 mM KOH. The pyridines were extracted with chloroform and analyzed from these tissues by procedures as described previously (13). Briefly, the homogenate was extracted rapidly with chloroform several times until minimal precipitate was observed at the interface of the buffer and chloroform. The supernatant was then passed through a 0.45 μm Ultrafilter-MC filtration device (Millipore, Bedford, MA) by centrifugation at 5000 rpm for 10 min at 4°C. This removed the residual DNA/protein and also served as a filter before the samples are loaded onto the HPLC column. The pyridine nucleotides were separated, analyzed, and quantitated using a C18 chromatography column (Waters Corp., Milford, MA) and HPLC (Waters Corp.). The mobile phase consisted of 50 mM potassium phosphate buffer (pH 7.05):acetonitrile (97:3) as suggested by the manufacturer.

Analysis of PCNA, Pu-1, C/EBPα, p53, and p73 in Bone Marrow. Wild-type and NQO1-null mice were sacrificed, and femurs removed by surgery and quickly frozen in liquid nitrogen. The ends of the bone were then removed under frozen conditions and flushed quickly with a ice-cold buffer containing 50 mM Tris-CI (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5% Triton X-100, and a mixture of protease inhibitors including 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 μg/ml each of pepstatin, aprotinin, leupeptin, benzamidine-CI, and antipain (all from Sigma Chemical Co.). Various bone marrow protein samples (75 μg) were separated on 10% SDS-PAGE, blotted on the ECL-membranes, and probed with antibodies against PCNA (PharMingen, San Diego, CA), myeloid cell differentiation factors Pu.1 (PharMingen), C/EBPα (a gift from Dr. Darlington Gretchen, Baylor College of Medicine, Houston, TX), tumor suppressor proteins p53 (ICN Biochemicals, Costa Mesa CA), p73 (a gift from Dr. Dennis Roop, Baylor College of Medicine), and tubulin (Sigma Chemical Co.). Western blots were developed with ECL (Amersham Pharmacia Biotech, Buckinghamshire, England) reagents by the procedures suggested by the manufacturer.

Apoptosis. Apoptosis was measured by using the annexin V-FITC detection kit (PharMingen). NQO1-null and wild-type mice were anesthetized, and bone was surgically removed. The bone marrow was flushed gently with sterile PBS. After two PBS washes, the cells were suspended in the annexin V-FITC binding buffer to a concentration of 1 × 10^6 cells/ml. Assay for the determination of apoptotic cells was essentially performed as described by the manufacturer and measured using Coulter (R) Epics XL-MCL Flow Cytometer (Beckman-Coulter Co, Miami, FL).

![Fig. 1. Characterization of NQO1-null mice.](image-url)
DISRUPTION OF NQO1 CAUSES MYELOGENOUS HYPERPLASIA

Table 1  Peripheral blood cell count of wild-type and NQO1–/– mice

<table>
<thead>
<tr>
<th>Blood cells</th>
<th>Wild type</th>
<th>NQO1–/–</th>
<th>Wild type</th>
<th>NQO1–/–</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC*</td>
<td>2.4 ± 0.7</td>
<td>1.6 ± 0.5 (0.025)</td>
<td>1.6 ± 0.7</td>
<td>1.0 ± 0.8 (0.025)</td>
</tr>
<tr>
<td>RBC*</td>
<td>8.9 ± 0.0</td>
<td>8.7 ± 0.3 (NS)</td>
<td>7.9 ± 0.4</td>
<td>8.4 ± 0.2 (NS)</td>
</tr>
<tr>
<td>Platelets*</td>
<td>1307 ± 139</td>
<td>1157 ± 317 (0.1)</td>
<td>1229 ± 17</td>
<td>1201 ± 136 (NS)</td>
</tr>
<tr>
<td>Neutrophils*</td>
<td>7.6 ± 4.3</td>
<td>17.5 ± 3.5 (0.005)</td>
<td>16.3 ± 5.0</td>
<td>34.2 ± 4.6 (0.005)</td>
</tr>
<tr>
<td>Eosinophils*</td>
<td>2.4 ± 1.8</td>
<td>3.2 ± 0.9 (0.1)</td>
<td>2.5 ± 1.5</td>
<td>3.2 ± 0.3 (0.01)</td>
</tr>
<tr>
<td>Basophils</td>
<td>0.4 ± 0.1</td>
<td>0.6 ± 0.4 (0.1)</td>
<td>0.4 ± 0.2</td>
<td>1.5 ± 0.8 (0.01)</td>
</tr>
<tr>
<td>Monocytes*</td>
<td>2.1 ± 0.4</td>
<td>2.0 ± 1.6 (NS)</td>
<td>2.5 ± 1.4</td>
<td>2.1 ± 1.4 (NS)</td>
</tr>
<tr>
<td>Lymphocytes*</td>
<td>87.5 ± 6.3</td>
<td>74.7 ± 6.6 (0.005)</td>
<td>76.6 ± 7.1</td>
<td>59.0 ± 5.5 (0.005)</td>
</tr>
</tbody>
</table>

* Mean ± SD of six mice.

Table 2  Myeloid and nonmyeloid cell ratio in the bone marrow

<table>
<thead>
<tr>
<th>Mice</th>
<th>Sex</th>
<th>Staining</th>
<th>Myeloid:Nonmyeloid ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Male</td>
<td>HEMA3</td>
<td>1.93 ± 0.53</td>
</tr>
<tr>
<td>NQO1–/–</td>
<td>Male</td>
<td>HEMA3</td>
<td>2.87 ± 0.44 (&lt;0.005)</td>
</tr>
<tr>
<td>Wild type</td>
<td>Female</td>
<td>HEMA3</td>
<td>1.83 ± 0.61</td>
</tr>
<tr>
<td>NQO1–/–</td>
<td>Female</td>
<td>HEMA3</td>
<td>2.21 ± 0.55 (&lt;0.005)</td>
</tr>
<tr>
<td>Wild type</td>
<td>Male</td>
<td>Myeloperoxidase</td>
<td>1.92 ± 0.25</td>
</tr>
<tr>
<td>NQO1–/–</td>
<td>Male</td>
<td>Myeloperoxidase</td>
<td>2.83 ± 0.20 (&gt;0.005)</td>
</tr>
<tr>
<td>Wild type</td>
<td>Female</td>
<td>Myeloperoxidase</td>
<td>1.20 ± 0.20</td>
</tr>
<tr>
<td>NQO1–/–</td>
<td>Female</td>
<td>Myeloperoxidase</td>
<td>1.85 ± 0.29 (&gt;0.005)</td>
</tr>
<tr>
<td>Megakaryocytes</td>
<td></td>
<td>(Percentage of total cells)</td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>Male</td>
<td>HEMA3</td>
<td>0.70 ± 0.10</td>
</tr>
<tr>
<td>NQO1–/–</td>
<td>Male</td>
<td>HEMA3</td>
<td>1.00 ± 0.15 (&gt;0.005)</td>
</tr>
<tr>
<td>Wild type</td>
<td>Female</td>
<td>HEMA3</td>
<td>0.68 ± 0.45</td>
</tr>
<tr>
<td>NQO1–/–</td>
<td>Female</td>
<td>HEMA3</td>
<td>1.40 ± 0.63 (&gt;0.005)</td>
</tr>
</tbody>
</table>

* Mean ± SD of six mice.

RESULTS AND DISCUSSION

Exon 6 of the NQO1 gene is the site for the P187S change in humans that abolishes NQO1 activity (5–8). By use of homologous recombination in embryonic stem cells, exon 6 was replaced with a neomycin cassette to generate NQO1-null mice (9). Neither NQO1 activity nor protein was detected in the various tissues examined, including the bone marrow (9; Fig. 1, A and B). Mice lacking NQO1 gene expression showed no detectable phenotype and were indistinguishable from the wild-type mice. The blood, urine, and serology analysis of wild-type and NQO1–/– mice did not show any sign of infection (data not shown).

The analysis of the peripheral blood from NQO1-null and wild-type mice revealed significant increases in the number of neutrophils, eosinophils, and basophils in NQO1-null animals (Table 1; Fig. 1C). NQO1-null mice had an increase in the number of segmented neutrophils (Fig. 1C). The number of platelets, RBCs, and monocytes showed no significant changes, whereas the WBCs and lymphocytes were lower in the NQO1-null mice. The decrease in the number of lymphocytes was significant, whereas the decrease in the total WBC count was marginal. The various changes were observed in 6-week-old mice, yet they were more prominent in 24-week old mice. Analysis of the bone marrow from NQO1-null mice revealed a significant increase in myeloid cells (Table 2; Fig. 2). This observation, along with the changes in the peripheral blood cell counts, demonstrates that loss of NQO1 results in myeloid cell hyperplasia, presumably caused by a shift from the lymphoid to myeloid lineage. The decrease in the total WBC counts may be caused by the significant reduction in lymphocytes. Interestingly, the bone marrow from NQO1-null mice also showed a significant increase in the number of megakaryocytes (Fig. 3). However, the increase in megakaryocytes did not cause an increase in the number of peripheral blood platelets (Table 1). The increase in megakaryocytes, without a corresponding change in peripheral blood platelets, suggests that the megakaryocytes may be undergoing apoptosis. This is a possibility because some of the megakaryocytes in NQO1-null mice appeared dysplastic (Fig. 3). Histological analysis of liver, kidney, spleen, and thymus did not reveal a difference between wild-type and NQO1-null mice (data not shown). The histological analysis of various tissues also did not show any sign of infection. This was also evident from the observation that total WBC count was normal in the blood of NQO1-null and wild-type mice (Table 1).

Both, wild-type and NQO1-null mice showed 40 acrocentric chromosomes in each metaphase spread, which is the diploid number of a laboratory mouse. No metaphases either from wild-type or NQO1-null mice contained obvious structural aberrations, even in G-banded preparations. A typical metaphase spread from a wild-type female, and a G banded karyotype from NQO1-null mouse is shown in Fig. 4. However, a few metaphases in both groups were tetraploid with a chromosome number 78–80. From these observations we conclude that NQO1-null mice did not contain any mitotic catastrophe, not even
in chromosome 2, which is heavily involved in radiation-induced myeloid leukemia (14). These results were not surprising, because \(NQO1\)-null mice are preconditioned but do not have myeloid leukemia. However, \(NQO1\)-null mice are susceptible, and expected to develop myeloid leukemia and associated chromosomal aberrations if exposed to radiations and/or leukemia-causing chemicals.

Two possible hypotheses were tested to investigate the mechanism of the role of \(NQO1\) in protection against myelogenous hyperplasia and megakaryocytosis in bone marrow. The first hypothesis proposes that loss of \(NQO1\) leads to an accumulation of endogenous quinone metabolites and increased oxidative stress, which causes an increase in myeloid cells and megakaryocytes. The second hypothesis proposes that loss of \(NQO1\) leads to an increase in the NAD(P)H:NAD(P) ratio, resulting in intracellular redox changes and an increase in myeloid cells/megakaryocytes.

\(NQO1\)-null and wild-type mice were given i.p. injections of oxidative stress-causing agent menadione, and analyzed for a menadione/oxidative stress effect on hepatic toxicity and hematopoiesis. The treatment of wild-type and \(NQO1\)-null mice with menadione resulted in hepatic damage as evident from elevation of hepatotoxicity markers aspartate aminotransferase and alanine aminotransferase in the serum of these mice (data not shown; Ref. 9). The hepatic damage was significantly more in \(NQO1\)-null mice, because of the absence of \(NQO1\), which detoxifies menadione. The mice livers, rich in cytochrome P450 reductase, catalyzes metabolic activation of menadione, leading to the generation of electrophiles and reactive oxygen species that cause hepatic damage. The \(NQO1\) enzyme in wild-type mice competes with P450 reductase, and catalyzes two-electron reduction and detoxification of menadione, thus leading to protection against menadione-induced hepatic damage. On the other hand, the \(NQO1\)-null mice deficient in \(NQO1\) and its protection against menadione demonstrated increased hepatic damage as compared with wild-type mice. Interestingly, no significant changes were observed between the untreated and menadione-treated wild-type and \(NQO1\)-null peripheral blood (Table 3) or bone marrow (data not shown). This may be related to lower amounts of one-electron reducing enzyme cytochrome P450 reductase in the bone marrow as compared with liver. These results suggested that oxidative stress is not a major factor underlying the myelogenous hyperplasia in the \(NQO1\)-null animals. However, it is not clear whether endogenous quinones and/or dietary quinones contribute to the myelogenous hyperplasia and increase in megakaryocytes.

Myelogenous hyperplasia was observed in the bone marrow of unchallenged \(NQO1\)-null mice. Therefore, it is likely that bone marrow cells from these mice accumulate NAD(P)H, the cofactor for \(NQO1\), thus changing the intracellular redox status of cells. Indeed, an analysis of the bone marrow revealed that the NAD(P)H:NAD(P) ratios were significantly higher in \(NQO1\)-null mice when compared with the wild-type mice (Fig. 5). In addition, the absolute levels of pyridine nucleotides (NAD(P)H and NAD(P)) were lower in \(NQO1\)-null mice presumably because the accumulation of NAD(P)H causes feedback inhibition of the pentose phosphate pathway, which provides

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**Fig. 3.** Bone marrow histology. \(NQO1\)-null and wild-type mice were sacrificed, and the femurs/sternums were surgically removed. The bones were fixed in 10% neutral buffered formalin and decalcified in 23% formic acid + 9% sodium citrate by procedures as described in “Materials and Methods.” The tissues were then embedded in paraffin and cut into 4-µm sections. The sections were placed onto slides and stained with H&E.

**Fig. 4.** A conventionally Giemsa-stained metaphase spread and a G banded karyotype from a wild-type female and \(NQO1\)-null male. A, metaphase plate from a wild-type mouse showing 40 acrocentric chromosomes. B, karyotype from a \(NQO1^{-/-}\) mouse showing normal G banding patterns.
ribose for the synthesis of NAD(P) (Fig. 5). These results in bone marrow were also supported by similar observations in the liver and kidney of NQO1-null mice as compared with wild-type mice (15). The liver and kidney of NQO1-null mice showed an increase in the NAD(P)H:NAD(P) ratio and decreased pyridine nucleotide levels (15). These led to alterations in the intracellular redox status and significant alterations in cellular metabolism of carbohydrates and fats (15). The NQO1-null mice showed lower blood levels of glucose, no change in insulin, and higher levels of triglycerides, β-hydroxy butyrate, pyruvate, lactate, and glucagon as compared with wild-type mice. An insulin tolerance test demonstrated that the NQO1-null mice have a mild insulin tolerance. The NQO1-null mice livers also showed significantly higher levels of triglycerides, lactate, pyruvate, and glucose. The liver glycogen reserve was found decreased in NQO1-null mice as compared with wild-type mice. The alterations in metabolism because of redox changes also resulted in a significant reduction in the amount of abdominal adipose tissue.

Western analysis failed to demonstrate a significant change in the levels of PCNA between the bone marrow from NQO1-null and wild-type mice (Fig. 6). Therefore, the contribution of cellular proliferation in myeloid cell hyperplasia of bone marrow in NQO1-null mice may be limited to none. Similarly, the two markers (C/EBPα and Pu-1) of myeloid cell differentiation also showed more or less no change between bone marrow of wild-type and NQO1-null mice indicating that differentiation processes may be functioning properly in bone marrow of NQO1-null mice (Fig. 6; data shown for Pu-1). This was also supported by the observations that neutrophils, eosinophils, and basophils all appeared well differentiated in peripheral blood of NQO1-null mice (Fig. 1).

NAD has been shown to serve as a substrate for poly(ADP-ribose) polymerase, a DNA binding protein that catalyzes the transfer of ADP-ribose residues from NAD to other proteins, including p53 (16). Therefore, we analyzed the levels of p53, its homologue p73, and apoptosis in the bone marrow of NQO1-null/wild-type mice. We found that p53, p73, and apoptosis were significantly lower in the NQO1-null mice (Fig. 7). The decrease in apoptosis in NQO1-null mice was significant (P > 0.01). These observations are in agreement with a recent report showing a decrease in the levels of p53 and apoptosis in cells that had been treated with an inhibitor (dicoumarol) of NQO1 (17). This report demonstrated that the decrease in p53 levels was through the proteasomal degradation pathway. Therefore, it is likely that the decreased levels of NAD(P), in NQO1-null bone marrow, results in degradation of p53, p73, and other yet unknown growth regulatory factors. The partial loss of p53 and p73 may lead to decreased apoptosis of myeloid cells in the bone marrow of NQO1-null mice. The role of NAD in stabilizing p53 is also suggested by other reports, which demonstrate decreased basal levels of p53 in human breast, skin, and lung cells grown in nicotinamide-deficient medium (18, 19). A decrease in apoptosis may account for the increase in myeloid cells in the NQO1-null bone marrow. The present studies using total bone marrow did not demonstrate a significant role for cellular proliferation and differentiation in NQO1-null mice. However, this needs to be confirmed by additional studies with more factors and markers, and isolated myeloid cells. Similarly, the roles of cellular proliferation, differentiation, and apoptosis in the increase of megakaryocytes in the NQO1-null bone marrow without a change in blood platelets levels also remain to be investigated.

The data presented above demonstrates that disruption of NQO1 causes an increase in myeloid cells and megakaryocytes. These results, together with previous reports that show a high frequency of the NQO1 P187S allele in patients with acute leukemias (7, 8), suggest that disruption of NQO1 predisposes people to develop acute myelogenic leukemia. This raises the question as to whether the NQO1-null mice are more susceptible to benzene-induced symptoms of acute leukemia.

Table 3
Peripheral blood cell count of 24-week-old wild-type and NQO1−/− mice treated with DMSO and menadione

<table>
<thead>
<tr>
<th>Blood cells</th>
<th>DMSO</th>
<th>Menadione</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>1.7 ± 0.6</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>RBC</td>
<td>7.9 ± 0.1</td>
<td>7.5 ± 0.5</td>
</tr>
<tr>
<td>Platelets</td>
<td>1473 ± 70</td>
<td>1471 ± 109</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>24.3 ± 6.6</td>
<td>23.7 ± 3.3</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>3.1 ± 1.2</td>
<td>3.2 ± 0.5</td>
</tr>
<tr>
<td>Basophils</td>
<td>0.4 ± 0.2</td>
<td>0.4 ± 0.3</td>
</tr>
<tr>
<td>Monocytes</td>
<td>2.1 ± 0.4</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>69.7 ± 5.4</td>
<td>28.3 ± 1.6</td>
</tr>
</tbody>
</table>

* Mean ± SD of six mice.
* Mean ± SD of six mice.
* Mean ± SD of six mice.

DISRUPTION OF NQO1 CAUSES MYELOGENOUS HYPERPLASIA

Fig. 5. NAD(P)/NAD ratio in bone marrow of wild-type (NQO1+/+) and NQO1-null (NQO1−/−) mice. Femurs were surgically removed and quickly frozen in liquid nitrogen to avoid changes in the levels of the pyridine nucleotides. Under frozen condition, the ends of the bone were cut and while the marrow was thawing, it was flushed with a solution containing KCN, bathophenanthroline, and KOH. The pyridines were extracted with chloroform and analyzed by HPLC procedures as described in “Materials and Methods.” The data are shown only for NADH/NAD.

A. Bone Marrow Cell Proliferation

PCNA

B. Bone Marrow Cell Differentiation

Pu-1

Fig. 6. Western analysis. Wild-type and NQO1-null mice were sacrificed and femurs were washed out. The bone marrow was flushed out from femurs and homogenized in an appropriate buffer containing protease inhibitors by procedures as described in “Materials and Methods.” Various bone marrow proteins (75 μg) were separated on 10% SDS-PAGE, blotted on the ECL membranes, and probed with antibodies against PCNA and C/EBPα. Western blots were developed with ECL (Amersham Pharmacia Biotech) reagents by the procedures suggested by the manufacturer.
myelogenous leukemias. Benzene exposure in humans is associated with aplastic anemia and AML (20–22). Benzene is metabolically activated by cytochrome P450 CYP2E1 to benzene oxide, which spontaneously forms phenol (23). Phenol is additionally metabolized to hydroquinone by CYP2E1 (24). Hydroquinone and related metabolites are converted in the bone marrow, by myeloperoxidase, to benzoquinones (25, 26). Benzoquinones are potent hematotoxic and genotoxic compounds (27, 28). Recently, bone marrow cells, including CD34+ progenitor cells homozygous for the P187S mutation, showed an increased susceptibility to hydroquinone/benzene toxicity (29, 30). Therefore, it is very likely that exposure of NQO1-null mice to chemicals such as benzene may lead to symptoms of acute myelogenous leukemia.

In addition to the increase in myeloid cells and megakaryocytes, the NQO1-null mice also exhibit a significant decrease in lymphocytes and a marginal decrease in WBCs. The marginally decreased WBC count may be because of a decrease in lymphocytes. However, it is not known whether the decrease in lymphocytes is directly related to the loss of NQO1. It is also possible that the increase in myeloid cells signals the down-regulation of lymphocytes to keep the total number of WBCs relatively constant. However, it is noteworthy that the low or null NQO1 P187S allele was found to be associated with chromosomal translocations and inversions in AML patients (31).

In conclusion, disruption of the NQO1 gene in mice causes myeloid hyperplasia in the bone marrow and an increase in megakaryocytes. Alterations in the NAD(P)H:NAD ratio and lower NAD levels lead to a decrease in the levels of the tumor suppressor proteins p53, p73, and apoptosis. These changes contribute to the increase in myeloid cells/megakaryocytes and have the potential to lead to AML in NQO1-null mice. This consequence of NQO1 disruption is in addition to its role in detoxification of redox cycling quinones and protection against their toxicity. These studies also suggest that human individuals lacking the expression of the NQO1 gene, caused by mutations such as the P187S, may be at higher risk for developing AMLs. The NQO1-null mice have great potential to develop into a model to study acute leukemias and anticancer chemotherapy.

ACKNOWLEDGMENTS

We thank our colleagues for helpful discussions. We also thank Robert Geske for help in HEMA3 and myeloperoxidase staining of bone marrow cells. The technical help provided by J. Haruhan for HPLC analysis is also greatly appreciated.

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