Reactive Oxygen Species, DNA Damage, and Error-Prone Repair: A Model for Genomic Instability with Progression in Myeloid Leukemia?

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Abstract

Myelodysplastic syndromes (MDS) comprise a heterogeneous group of disorders characterized by ineffective hematopoiesis, with an increased propensity to develop acute myelogenous leukemia (AML). The molecular basis for MDS progression is unknown, but a key element in MDS disease progression is loss of chromosomal material (genomic instability). Using our two-step mouse model for myeloid leukemic disease progression involving overexpression of human mutant NRAS and BCL2 genes, we show that there is a stepwise increase in the frequency of DNA damage leading to an increased frequency of error-prone repair of double-strand breaks (DSB) by nonhomologous end-joining. There is a concomitant increase in reactive oxygen species (ROS) in these transgenic mice with disease progression. Importantly, RAC1, an essential component of the ROS-producing NADPH oxidase, is downstream of RAS, and we show that ROS production in NRAS/BCL2 mice is in part dependent on RAC1 activity. DNA damage and error-prone repair can be decreased or reversed in vivo by N-acetyl cysteine antioxidant treatment. Our data link gene abnormalities to constitutive DNA damage and increased DSB repair errors in vivo and provide a mechanism for an increase in the error rate of DNA repair with MDS disease progression. These data suggest treatment strategies that target RAS/RAC pathways and ROS production in human MDS/AML.

Introduction

It is now well established that the progression of normal cells to neoplastic transformation results from the accumulation of mutations in genes that control cellular proliferation, survival, and differentiation. Approximately, 30% myelodysplastic syndrome (MDS) cases progress to acute myelogenous leukemia (AML; refs. 1, 2). Although little is known of the genetic basis of MDS, several genetic alterations have been noted. A significant percentage of MDS cases are characterized by chromosomal deletions of 5q or 7q (3), and has previously been reported to be high in a proportion of myeloid malignancies (4, 5). The next most frequent genetic alteration in MDS is activating mutations of the RAS homologues occurring in ~20% of MDS patients reviewed in refs. (6, 7). In addition, the decrease in the rate of apoptosis with disease progression correlates with increases in intracellular levels of BCL2 family proteins (8). Activation of both RAS and BCL2 genes has previously been reported to be in high proportion with myeloid malignancies (reviewed in ref. 9). Activating mutations of NRAS and Kras occur in some 30% of chronic myelomonocytic leukemia and AML patients (6), and BCL2 has been shown to be up-regulated in 50% of AML with poor prognosis (10).

The alteration of these genes suggests candidates for development of mouse models for myeloproliferative disease (MPD)/MDS/AML. Mutant RAS has been shown to give rise to MPD (11, 12), and BCL2 accelerates disease in both myeloid and lymphoid disorders (13). We created transgenic mice bearing mutant NRAS and BCL2 driven by the MRP8, which directs expression of the transgene to committed myeloid progenitors and neutrophils. NRAS mice have a relatively mild phenotype with an increase of immature neutrophils (14). The BCL2 mice seem to have an increase in marrow blasts, but nevertheless have normal peripheral blood cell counts (15). Crossing transgenic mice harboring mutant NRAS and BCL2 genes results in a lethal transplatable disease developing in 2 to 6 months with a phenotype morphologically resembling human AML with dysplasia (FAB; ref. 16) with increased marrow blasts and invasion of tissue organs (Fig. 1; Supplementary material). These mice have reduced peripheral blood platelet counts, splenomegaly, and an expansion of the primitive myeloid progenitor (Sca-1+) population with increased RAS and BCL2 associations and RAS activity.

Cells in many or all cancers are genomically unstable, leading to the accumulation of gene abnormalities with time, which are thought to cause disease progression (17). Although the mechanisms remain elusive, it is widely recognized that genomic instability accentuates the acquisition of molecular abnormalities (the mutator phenotype theory; ref. 17). In normal, nonmalignant cells, there are pathways that maintain genomic integrity by repairing DNA damage without causing deletions or mutations (18). Double-strand breaks (DSB) are considered the most lethal...
form of DNA damage and can occur as a result of endogenous insults, such as attack by oxygen radical species produced during metabolism or from exogenous insults, such as ionizing radiation. Mammalian cells have evolved sophisticated mechanisms for detecting and repairing DSB. There are at least two mechanisms for the repair of DSB, homologous recombination (HR) and non-homologous end-joining (NHEJ). HR is an error-free repair pathway (19), whereas NHEJ is an error-prone repair pathway. NHEJ has been extensively characterized in rodent cells (reviewed in ref. 20), identifying a pathway in which the subunit proteins of the Ku70/Ku86 heterodimer bind free DNA ends at the sites of DSB and are then phosphorylated by DNA-protein kinase (DNA-PK), which is activated by the bound Ku70/Ku86 heterodimer (21, 22). The targeted free ends are subsequently ligated by DNA ligase IV in conjunction with XRCC4 (23). However, although NHEJ can repair breaks in an error-free manner, it can also introduce errors in the form of small DNA deletions of <30 bp during repair (24). Such errors, generated at an increased frequency, could contribute to the accumulation of mutations (these refer broadly to genomic alterations) in these cells (25, 26). Previously we found that myeloid malignancies were characterized by significantly increased mis-repair frequencies compared with normal human myeloid progenitors (CD34+) cells. Importantly, the mis-repair was characterized by large plasmid deletions (>250 bp) in our editors (CD34+) cells. Importantly, the mis-repair was characterized by increased error-prone NHEJ in preleukemic disease with a dramatic increase in the blast percentage of bone marrow blast cells.

Figure 1. Model of leukemia progression. Preleukemic disease is initiated by one or more genetic events in a bone marrow CD34+ progenitor cell. Expansion of this abnormal clone occurs. Disease progression involves additional genetic alterations. Transgenic NRAS mice have a mild phenotype, a small increase in blasts and no invasion of the liver. NRAS-BCL2 cells have a progressed disease with a dramatic increase in the blast percentage of bone marrow blast cells.

Materials and Methods

Transgenic mice. MRP8BCL2-2 (15) and MRP8NRASD12 (14) mice were obtained from their respective originators. The animals were maintained in the barrier facilities of the University of California, San Francisco and the Institut Universitaire d’Hematologie, Paris, under the appropriate animal project licenses. The mice were bred and genotyped using standard husbandry and PCR techniques as described previously (13).

For antioxidant administrations, mice were given N-acetyl cysteine (40 mmol/L; Sigma) in their drinking water for a period of 6 weeks postgestation, according to the protocols of Reliene et al. (30). This same intake of N-acetyl cysteine was shown to reduce DNA adduct formation in rats exposed to genotoxic agents (31). Offspring gestated in N-acetyl cysteine environment were then tested for genomic instability as detailed below. The control group received acidic (pH 2.8) drinking water.

Tissue preparation. Blood was obtained from anaesthetized animals (with isoflurane) by venipuncture of retroorbital vein plexus. Bone marrow was obtained by flushing long bones with Hank’s balanced salts solution followed by filtering through nylon mesh. Spleen cells were prepared by gently crushing the organ with a syringe plunger, and single-cell suspensions were obtained by filtering through nylon mesh. Spleen cells were prepared by gently crushing the organ with a syringe plunger, and single-cell suspensions were obtained by filtering through a wire mesh. Sca-1+ and Mac-1+ (CD11b) fractions were separated using an AutoMacs separator (Miltenyi). Lineage negative (Lin−) cells were isolated from pooled bone marrow of three to four mice using a cocktail of the following antibodies coupled to microbeads: Mac-1, Gr-1, Ter-119, CD45R, CD5. The cells were passed through an AutoMacs. The Lin− cells were then marked with anti-Sca-1 antibody coupled to PE and anti-KIT antibody coupled to APC and were sorted to give rise to Lin−/Sca-1−/Kit− cells.

Preparation of nuclear extracts. Nuclear extracts were prepared based on the Sigma protocol (Sigma Aldrich). Dialysis of the nuclear extracts was for 2 × 3 h against E buffer [20 mmol/L Tris-HCl (pH 8.0), 0.1 mmol/L KOAc, 20% (v/v) glycerol, 0.5 mmol/L EDTA, and 1 mmol/L DTT; Sigma Aldrich]. Finally, nuclear extracts were fast frozen and stored at −80°C.

Plasmids and antibodies. pUC18 was linearized with EcoR1 (MBI Fermentas), dephosphorylated with calf intestine alkaline phosphatase (Promega) and 32P-labeled with T4 polynucleotide kinase (Promega). Rabbit polyclonal antisera raised against Ku86, Ku70, and DNA-PKcs were purchased from Serotec. Rabbit anti-yH2AX and mouse anti-phosphorylated ATM were purchased from Upstate.

Plasmid reactivation assay. Nuclear extracts were prepared as for the end-ligation assay. We used the DNA repair fidelity assay devised by North...
et al. (32). For the assay, 1 μg of EcoRI linearized pUC18 was incubated with 6 μg of nuclear extract. Reactions (10 μL) were carried out in 50 mM L-triethanolamine-HCl (pH 7.5), 60 mM L-KOAc, 50 μM L-deoxyribose- 5-tide triphosphate, 0.5 mM L-MgAc, 2 mM L-ATP, 1 mM L-DTT, and 100 μg/mL bovine serum albumin (BSA). The mixture was incubated for 24 h at 18°C. The plasmid was purified from the extract by passing it down a filter column and then diluted threefold in TE buffer before being used to transform Escherichia coli strain DH5a. Transformed cells were plated out on LB agar plates, including 50 μg/mL ampicillin, 2% X-gal, and 10 mM L-isopropyl-thio-B-O-galactopyranoside. The plates were incubated at 37°C for 17 h, and then placed at 4°C for 3 h before colonies were counted. To allow for spontaneous rejoining/incomplete EcoRI cutting, assay controls were conducted without nuclear extract. The number of colonies generated was subtracted from extract-treated plasmid colonies. In addition, to correct for bacterial plating numbers and to determine whether nuclease activity was affecting plasmid efficacy, cells were plated on LB agar without ampicillin. Primers around the EcoRI site were designed to give a PCR product of 628 bp corresponding to nucleotides 150 to 777 bp.

In addition, to correct for bacterial plating numbers and to determine whether nuclease activity was affecting plasmid efficacy, cells were plated on LB agar without ampicillin. Primers around the EcoRI site were designed to give a PCR product of 628 bp corresponding to nucleotides 150 to 777 bp. Colony PCR was done on blue and white colonies to determine the size of the deletion. For in vivo plasmid reactivation studies, linearized plasmid was transfected into log-phase cells at 1 × 10^7/mL using Fugene reagent (Invitrogen). Cells were left for 24 h before plasmid extraction using miniprep columns (Quagen). Plasmid reactivation was then carried out as stated before.

Chromatin fibers. Chromatin fibers were prepared from 1 × 10^6 cells according to the protocols of Raderschall et al. (33). However, the technique was modified to ensure subsequent detection of proteins binding at specific points of interest. Proteins were cross-linked to DNA by adding formaldehyde (1% final concentration) to the culture medium for 10 min at 37°C. Aliquots of 1 × 10^6 cells were cytopsin onto glass slides and covered with 50 μL of 50 mM L-Tris-HCl (pH 8), 1 mM L-EDTA, and 0.1% SDS. After 1-min incubation with this detergent solution, the chromatin was mechanically sheared on the slide with the aid of a glass coverslip, then fixed in ethanol for 30 min at −20°C, and then rinsed in ice-cold acetone for up to 1 min.

Immunofluorescence. Slides with chromatin fibers were incubated with blocking solution (10% BSA/4× SSC/0.1% Tween 20) for 30 min at 37°C. Thereafter, slides were incubated in primary antibody diluted in blocking solution (1:10 to 1:50) and incubated for 30 min at 37°C. Slides were washed for 5 min in 4× SSC/0.1% Tween 20, and this was repeated twice more. The blocking step was repeated before slides were incubated in secondary antibody conjugated with fluorochromes diluted in blocking solution and then fixed in ethanol for 30 min at −20°C, and then rinsed in ice-cold acetone for up to 1 min.

DNA damage studies. Cells were grown in 10 mmol/L BrdUrd (Sigma Aldrich) for ~30 h. Flasks were shielded from light. Thereafter, the cells were washed and placed in BrdUrd-free medium for 1 h. Chromatin fibers were prepared from 1 × 10^6 cells, according to protocols of Raderschall et al. (33). However, we modified the protocol to ensure subsequent detection of proteins binding at specific sites of interest. Proteins were cross-linked to DNA by adding formaldehyde (1% final concentration) to the culture medium for 10 min at 37°C before harvesting. Aliquots of 1 × 10^6 cells were cytopsin onto glass slides and covered with 50 μL of 50 mM L-Tris-HCl (pH 8), 1 mM L-EDTA, and 0.1% SDS. After 1-min incubation with the detergent solution, the chromatin was mechanically sheared on the slide with the aid of a glass coverslip and then fixed with methanol and acetone. Antibodies to BrdUrd will only detect BrdUrd incorporated at ss-DNA regions. Thus, this protocol is specific for the detection of regions of DNA damage.

Reactive oxygen species measurement. Cells (1 × 10^6) were incubated with 10 mmol/L fluorescent probe dihydrodorhodamine-123 for 20 min at 37°C. Cells were washed twice and resuspended in buffer for flow cytometry (PBS/1% BSA/10 mM EDTA/0.1% NaN3). Relative levels of reactive oxygen species (ROS) were measured in arbitrary units of mean fluorescence intensity in the FL-1 channel with respect to untreated controls [without H2O2 and with H2O2 (1 mM)] using FACSCalibur and Cell Quest Software, Becton Dickinson.

Inhibitor assays and RAC1-GTP pull-down. Dose and time course RAC1 inhibition of Mac-1+ and B220+ sorted cells were optimized and reproducible at incubation for 16 h at 37°C with 5% CO2 (DMEM/10%FCS) with 5 or 50 μM of the RAC1 inhibitor (NSC27366 in DMSO vehicle; kindly provided by David A Williams, Cincinnati, OH), followed by ROS measurement and RAC1 pull-down assay. For RAC1 pull-down assays, cells were lysed in 50 mM L-Tris (pH 7.4), 1% NP40, 15% glycerol, 200 mM L-NaCl, 5 mM L-MgCl2, 5 mM NaF, 1 μM L-leupeptin, 0.1 μM L-protinin, and 1 mM L-phenylmethylsulfonyl fluoride. Detergent-insoluble material was removed by centrifugation (16,000 × g at 4°C for 20 min). Activation-sensitive pull-down assays were carried out using the binding domains of PAKs (amino acid 1–252; kindly provided by Chris J Marshall, London, United Kingdom) as previously described (34) and assessed by Western blotting (12% SDS-PAGE). Comparative total protein content was determined by parallel Western blotting of the whole-cell lysate; both probed with the RAC1-specific antibody (Upstate Biotechnology) at 4°C overnight, horseradish peroxidase–conjugated secondary antibody probing for 1 h at room temperature, and detection using ECL Plus reagents (Amersham Pharmacia) according to the manufacturer’s instructions.

**Results**

To determine whether there is an increase in DNA damage and aberrant repair during leukemia progression, we studied a mouse model for myeloid leukemic disease progression involving over-expression of the mutant NRAS and BCL2 genes that we have established (Fig. 1). Double transgenic NRAS/BCL2 mice have AML with evidence of dysplasia or myeloid leukemia without maturation. Transgenic mice bearing NRAS driven by the MRP8 promoter, which directs expression of the transgene to committed myeloid progenitors and neutrophils, have a relatively mild phenotype with an increase of immature neutrophils. The BCL2 mice seem to have an increase in marrow blasts but are nevertheless hematologically normal. Crossing transgenic mice harboring NRAS and BCL2 genes results in a heterogeneous disease phenotype, morphologically resembling human AML with evidence of dysplasia indicative of a preceding MDS with increased marrow blasts and invasion of tissue organs (Fig. 1).

We assayed for DNA damage in bone marrow cells from NRAS and BCL2 single transgenic mice, using an established immunofluorescence-based assay for ss-DNA damage, dependent on BrdUrd incorporation (33). This assay relies on the fact that anti-BrdUrd antibodies can only detect BrdUrd incorporated into DNA when it is in single-stranded form. We and others have previously shown that these regions of DNA damage can detect nonblunted DSB by showing that several DSB repair proteins were colocализed to these regions (26, 33, 35). Upon examination of BrdUrd-incorporated chromatin fibers in bone marrow mononuclear cells (BMC) from both NRAS and BCL2 mice, we saw that NRAS and to a lesser extent BCL2 show an increase in the frequency of constitutive DNA damage compared with normal mice (NRAS 35% versus 8%, mean n = 3), (BCL2 15% versus 8%, mean n = 3). Interestingly, the pattern of DNA damage along chromatin fibers was different for NRAS cells compared with those from BCL2 transgenic mice. NRAS gave staining along the length of the fiber whereas in BCL2 fibers, the staining was more punctate.
we showed that the mean number of mice have increased foci above that of the normal FVBN mice. In compared with cells have increased numbers of NRAS+BCL2 damage and repair showed in chromatin fibers. We find that the fibers were examined from, confirming the increased DNA BrdUrd (Fig. 2A). An average of 18 chromatin fibers examined from three separate experiments with mononuclear cells from spleen and bone marrow of BrdUrd-incorporated NRAS transgenic mice cells, 85% of fibers with signal show colocalization to γH2AX (Supplementary Table S1). We further determined that ATM is known to be phosphorylated in response to DSB and, to bind to chromatin in the region surrounding the DSB damage (37), colocalizes with γH2AX (Fig. 2A). The formation of γH2AX foci in nuclei, following DNA damage, has been used as a quantitative measure of DSB damage and repair. We determined whether we could also detect quantitative differences in γH2AX foci formation in the nuclei of cells from NRAS versus NRAS+BCL2 transgenic mice from the same samples the fibers were examined from, confirming the increased DNA damage and repair showed in chromatin fibers. We find that NRAS+BCL2 cells have increased numbers of γH2AX foci compared with NRAS, and indeed cells from both transgenic mice have increased foci above that of the normal FVBN mice. In an average of 34 cells examined from three experiments done, we showed that the mean number of γH2AX foci per cell from normal FVBN spleen and bone marrow was 2 ± 2, whereas cells from BCL2 and NRAS transgenic mice gave 4 ± 2 and 10 ± 2 γH2AX foci, respectively. Importantly, spleen and bone marrow cells from double transgenic mice gave an increased number of γH2AX foci per cell and above that for both normal and single transgenic mice at 18 ± 2 foci (Supplementary Table S3).

To determine whether the error-prone NHEJ pathway participates in the repair of the DSB that we have detected, we did coimmunostaining of these regions of DNA damage with antibodies to protein components from the NHEJ repair pathway on chromatin fibers. We find that DNA damage colocalizes with the NHEJ protein Ku86 (Supplementary Table S4). In an average of 13 fibers examined from three separate experiments, 75% and 86% of fibers with BrdUrd staining from NRAS and NRAS+BCL2 transgenic BMC show colocalization with Ku86, respectively. Furthermore, we also found that γH2AX colocalizes with Ku86 (data not shown). These data emphasize that DNA damage is linked to repair by NHEJ in situ.

Next, we sought to determine whether we could detect altered NHEJ repair activity that might be activated by the constitutive DNA damage we have shown. We have previously shown that myeloid leukemia cells contain increased repair infidelity of the NHEJ repair pathway for DSB (25–27). Therefore, we determined whether NHEJ repair fidelity was altered in BMC from the NRAS and BCL2 transgenic mice, using an in vitro plasmid end-joining assay in cell-free extracts. Nuclear extracts were prepared from bone marrow and spleen cells and used in a lacZ plasmid reactivation assay; this colony assay allowed quantitation of correctly repaired colonies (blue) versus incorrectly repaired (white) colonies (32). Figure 3A shows that BMC from single transgenic mice (NRAS, BCL2) had an increased misrepair frequency (NRAS 7.6% versus 3.9%, BCL2 6.5% versus 3.9%, n = 3) compared with normal FVBN cells. White colonies were randomly
chosen from the test plates and were analyzed for plasmid deletions using PCR. NRAS and to a lesser extent BCL2 cells elicited a higher percentage of large plasmid deletions (35–400 bp) compared with FVBN cells (<30 bp, NRAS 55% versus 10%, BCL2 45% versus 10%, n = 3; Fig. 3A; Supplementary Table S5).

We next determined whether the increased ss-DNA and DSB that accompany MDS disease progression in the NRAS+BCL2 double transgenic mice are associated with further increases in errors of end-joining. Nuclear extracts were examined from the spleen and bone marrow cells of those double transgenic mice that showed disease progression as determined by platelet counts of below 800,000/μL, bone marrow smears showing high blast counts of 50% to 90%, and histologic sections showing invaded livers and spleens. These mice show a large and significant increase in misrepair frequency (25.9%, n = 3, P < 0.001) compared with both the single transgenic and normal controls (Fig. 3A). This is also reflected in the increase of large plasmid deletions detected from in vitro plasmid rejoining assays (70% versus 10%, n = 3, P < 0.001; Fig. 3A).

In general, double transgenic mice that did not show disease progression did not exhibit increased misrepair frequencies (data not shown).

If the misrepair frequencies are related to myeloid disease progression, then the differences should be manifested in the myeloid cell compartment. We next determined whether the
altered misrepair frequencies were detected in the myeloid compartment as opposed to the lymphoid cell compartment. Myeloid (Mac-1+; Sca-1+) and B-lymphoid (B220+) cells were purified from the spleens of transgenic and normal control mice, and plasmid reactivation assays were done. Myeloid cells in FVBN mice show a small increase in end-joining misrepair frequencies compared with lymphoid cells (Mac-1+ 7.1%, Sca-1+ 7.2% versus B220+ 3.6%). However, in NRAS+BCL2 cells, misrepair frequencies in myeloid cells were significantly increased (Mac-1+ 22.3% versus B220+ 6.2%, P < 0.05; Fig. 3B).

Previous studies have shown that cell lines transduced with activating HRAS mutants show increased production of ROS (38). Furthermore, it is now well established that in addition to the formation of 8-oxoguanine adducts, ROS are an endogenous source for ss-DNA and DSB (18, 39). We, therefore, tested whether NRAS also causes increased ROS production in NRAS/BCL2 transgenic mice by staining cells with the fluorescent probe dihydrorhodamine-123. This compound is oxidized to the fluorophore rhodamine-123 by ROS with absorption/emission maxima of 507:529 nm and was thus detected by flow cytometric analysis (40). The integrity of the assay is shown via the increased ROS production in myeloid progenitor cells (FDCP1 cell line) after H2O2 treatment, wherein H2O2 is a well established producer of ROS (41). We find that, as expected, H2O2 produces an increase in ROS above baseline levels (Fig. 4A). BMC from NRAS mice show increased ROS compared with FVBN mice (P < 0.05; Fig. 4B). Further increases in ROS above that exhibited by NRAS mice are seen in the NRAS+BCL2 double transgenic (P < 0.05; Fig. 4B and C). Importantly, we find that ROS levels are increased significantly in the myeloid compartment of NRAS+BCL2 double transgenic mice compared with the lymphoid (B cell) compartment (Fig. 4D).

We wished to examine the effect that decreasing ROS activity would have on DNA damage and repair in MDS/MPD mice in vivo. It is well known that ROS levels can be reduced by treatment with antioxidants, such as N-acetyl cysteine (31). First, we determined whether the increased DSB we reported in NRAS/BCL2 transgenic animals could be reduced or reversed. Normal and NRAS and NRAS+BCL2 transgenic mice were fed N-acetyl cysteine (40 mmol/L; Sigma) in their drinking water for a period of 6 weeks to the end of gestation according to the protocols of Reliene et al. (30), and offspring were examined for DNA damage and repair. This same

**Figure 4.** A, ROS measurement of myeloid progenitor, FDCP 1 cells treated with H2O2 by flow cytometry, using dihydrorhodamine-123. Increasing fluorescence due to ROS (y axis) is plotted against the cellular forward scatter (x axis). These are typical plots of ROS production in FDCP 1 with and without H2O2 treatment (n = 3 experiments). B, ROS measurement in BMCs from transgenic mice and wild-type (FVBN) controls, wherein significant difference to FVBN is denoted by * (P < 0.05). C, representative plot of ROS activity. Solid line, FVBN; dotted line, NRAS; shaded, NRAS+BCL2. D, relative levels of ROS in myeloid progenitor (Mac-1+) cells versus lymphoid (B220+) progenitors in NRAS+BCL2 mice.
intake of N-acetyl cysteine was shown to reduce DNA adduct formation in rats exposed to genotoxic agents (31). The control group received acidic (pH 2.8) drinking water. We examined H2AX foci formation in N-acetyl cysteine–treated and control age-matched untreated animals directly thereafter. We found a 30% to 50% reduction in the frequency and staining intensity of H2AX foci in N-acetyl cysteine–treated animals compared with untreated controls (Fig. 5A). To determine whether N-acetyl cysteine treatment also resulted in a reduction of DNA damage in the stem cell compartment, we measured H2AX foci in lin−Sca-1+ Kit+ cells from N-acetyl cysteine–treated animals and untreated controls. We find that DNA damage foci are reduced ~50% compared with controls (Fig. 5B). These data indicate that the DNA damage data derived from the myeloid compartment are representative of DNA damage in the stem cell compartment. DNA damage caused by NRAS and NRAS+BCL2 in vivo is dependent on ROS production. We next examined NHEJ misrepair activity in N-acetyl cysteine–treated transgenic and control mice. We find no significant decrease in NHEJ misrepair activity after N-acetyl cysteine treatment. However, BMC from double transgenic NRAS+BCL2 mice treated with N-acetyl cysteine mice showed a small but reproducible decrease in the frequency of NHEJ misrepair (Fig. 5C, top). Interestingly, however, the size of plasmid deletions examined from plasmid reactivation experiments showed a significant decrease in size (Fig. 5C, bottom) after N-acetyl cysteine treatment (e.g., large deletion percentage, NRAS+BCL2 75% versus NRAS+BCL2 + N-acetyl cysteine 40%, P < 0.05). Therefore, both in vivo DNA damage and NHEJ misrepair activity caused by NRAS and NRAS+BCL2 seem in part dependent on the production of ROS. However, this benefit did not translate into improved survival (data not shown).

We next examined the underlying mechanisms responsible for production of ROS in NRAS+BCL2 mice. Within the context of myeloid dysregulation, we have previously shown the role of RAC-mediated signaling pathways (34). In addition, increased ROS may be produced by RAS signaling through RAC, a downstream target of RAS activation (42). Consistent with the flow cytometry data (Figs. 4B–D and 6A), increased levels of RAC1 activity were observed within the Mac-1+ compartment compared with B220+ in NRAS+BCL2 double transgenic mice (Fig. 6B, top, lane 1 versus lane 4). Therefore, we next determined whether the increased ROS detected in the myeloid compartment of NRAS+BCL2 mice could be diminished by inhibition of RAC1. Thus, myeloid (Mac-1+) and lymphoid (B220+) cells were purified from the spleens of NRAS+BCL2 double transgenic and wild-type mice, incubated with the highly selective cell-permeable RAC1 inhibitor (NSC23766), and then followed by ROS measurement (43). We find that the RAC1 inhibitor has a marginal effect on ROS levels in Mac-1+ cells from FVB/N mice, and reduced levels are observed in the B220+ cells of RAS+BCL2 mice. In addition, insignificant change in ROS is seen in Mac-1+ cells from NRAS mice (data not shown). However, the RAC1 inhibitor has significant and titerable effect on the ROS activity in Mac-1+ sorted NRAS+BCL2 double transgenic cells (Fig. 6A). These data are confirmed with RAC1-GTP pull-down assays from NRAS+BCL2 transgenic mice (Fig. 6B). Whereas RAC1 activity is not significantly changed in B220+ cells after exposure to the RAC1 inhibitor or Mac-1+ cells from single transgenic animals (Supplementary Fig. S1), Mac-1+ cells from NRAS+BCL2 mice show a dramatic decrease in RAC1-GTP in the presence of NSC23766 (Fig. 6B).

**Discussion**

Several lines of evidence suggest that the induction and repair of DSB are critically involved in the acquisition of genomic instability (18). One scenario for genomic instability may manifest itself through increased induction of DSB in a cellular environment of compromised DSB repair. We previously described constitutive DNA damage and inaccurate repair of DSB in myeloid leukemias and proposed that these processes drive genomic instability in these malignancies (25, 26, 28). However, little is known of the mechanism(s) that may lead to an increase in genetic alterations with cancer or leukemia progression.

Studies have shown the activation of RAS and its downstream effector pathways in human MDS and AML (reviewed in ref. 44). In addition, overexpression of BCL2 is also observed in a variety of myeloid malignancies (9). Here, we have shown that in a two-step mouse model of myeloid leukemia disease progression involving overexpression of the mutant NRAS and BCL2 oncogenes, tumor progression is accompanied by an increase in ROS, which leads to an increased DSB and inaccurate repair by the error-prone NHEJ pathway. Thus, activation of oncogenic changes can initiate a cycle of genomic instability that has the potential to create further mutations, which in turn may facilitate leukemic disease progression.

It is well established that activation of oncogenes can lead to ROS production (45), and ROS is an established source of endogenous DSB (18). Several lines of evidence now indicate that activation of RAS–mitogen-activated protein (MAP) kinase pathways can generate increased ROS. HRAS increases ROS production and BCR-ABL has been shown to cause constitutive DNA DSB and improper repair through increased ROS production (29, 38, 46). Data presented here show that mutant NRAS causes an increase in ROS levels, and ROS levels are increased further in NRAS+BCL2 double transgenic mice that have progressed myeloid disease. In keeping with myeloid disease of these mice, these above alterations are seen in myeloid (Mac-1+ and Sca-1+ progenitor) versus lymphoid (B220+) cells. Although the mechanisms by which activation of BCL2 in these mice contributes to production of ROS is unclear, one explanation for the further increase in ROS in NRAS+BCL2 cells may be the antiapoptotic activity of BCL2, which would increase survival of those cells that have high NRAS expression. As RAS and BCL2 cooperate resulting in increased ROS activity (Supplementary material). BCL2 may also mediate increased ROS by increasing ROS activity and signaling to downstream effectors. We have excluded that BCL2 on its own could contribute to ROS production. Thus, increased ROS production, in part through RAS-RAC pathway, followed by misrepair of DSB, as shown by increased immunostaining of γH2AX and phosphorylated ATM, could represent an important general mechanism for creating genomic instability during the progression of human tumors.

Our findings of decreased DNA damage with antioxidant treatment confirm that at least a large proportion of the DSB detected in the cells of our NRAS and NRAS+BCL2 transgenic mice are directly produced by ROS. Although we found a significant decrease in DNA DSB as measured by γH2AX foci after N-acetyl cysteine treatment, this did not lead to a concomitant correction of repair at DSB or rescue of disease onset with improved survival. However, antioxidant treatment significantly decreased the size of deletions at repaired DSB. We have previously reported large deletions in *in vitro* assays for
NHEJ in nuclear extracts from myeloid malignancies (25) compared with normal CD34+ cells from normal individuals, and we suggested that these deletions may reflect aberrant exonuclease activity at DNA DSB. These data suggest that antioxidant treatment may lead to decreased aberrant processing at DSB ends (25). Thus, N-acetyl cysteine treatment may counteract some, but not all, of the altered activities that drive disease progression in myeloid malignancies.

Figure 5. A, γH2AX foci in nuclei from NRAS, NRAS+BCL2, and wild-type (FVBN) BMC with and without N-acetyl cysteine treatment. Nuclei (blue) with DAPI and nuclear foci (red). Foci ± SD from \( n = 3 \) experiments. B, γH2AX foci in nuclei from NRAS, BCL2, and wild-type (FVBN) bone marrow Lin−−Sca-1+KIT+ with and without N-acetyl cysteine treatment. Nuclei (blue) with DAPI and nuclear foci (green). Foci ± SD from \( n = 1 \) experiments. C, antioxidant pretreatment reduces error-prone repair. Top, mean misrepair frequencies (\( n = 3 \)). Filled-in columns, untreated mice; striped columns, N-acetyl cysteine–pretreated mice; bars, SE. Bottom, plasmid ligation assay. PCR of white colonies. Lanes 1-16, colony PCRs from NRAS spleen cell nuclear extracts; lanes 16-38, colony PCRs from N-acetyl cysteine–pretreated NRAS spleen cell nuclear extracts; lanes 39-51, colony PCRs from NRAS+BCL2 spleen cell nuclear extracts; lanes 52-74, colony PCRs from N-acetyl cysteine–pretreated spleen cell NRAS+BCL2 Mac-1+ fraction nuclear extracts. L, ladder; B, blue colony (628 bp).
Several lines of evidence suggest that activation of one or more of the subpathways of RAS-MAP kinases are critical in the production of ROS. One candidate pathway for ROS production in MDS may be signaling through RAC1 (47). Several groups have reported that RAC activation of NADPH oxidase is not limited to phagocytes (48). Irani et al., among others, found that dominant negative RAC1 blocked ROS production in HRAS-containing fibroblasts (38). Another candidate pathway for ROS production is signaling through extracellular signal-regulated kinase 1/2 (ERK1/2). ROS was shown to be produced by signaling through ERK1/2 in neuroblastoma cells (49). However, in corroboration with our previous findings in the context of myelopoietic cells (34), we find that in the myeloid (Mac-1+) cells from N-RAS+BCL2 ROS production is at least in part via the RAC1-mediated signaling pathway, with increased RAC1-activity in this compartment and reduced ROS levels with RAC1 inhibition. Thus, these results point to future therapies involving inhibition of ROS through the blocking of critical RAS-RAC1 signal transduction pathways in combination with general antioxidant treatment, such as N-acetyl cysteine. These therapeutic options are likely to represent important treatments in MDS/AML. Nevertheless, efficacy of ROS reduction on the reversal of genomic instability and disease progression may rely on elucidation of the major routes for ROS overproduction in cancer with multiple genetic alterations.

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