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GENETIC INSTABILITY AS AN AREA OF POTENTIAL THERAPEUTIC INTERVENTION

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Abstract Genomic instability is a characteristic feature of myeloid malignancies and the preleukemic syn dromes that predispose to these leukemias. We have accrued evidence in a transgenic mouse model for myeloid disease progression that genomic instability in myeloid malignancies may be driven by a combination of ongoing constitutive DNA damage coupled with altered repair of double strand breaks (DSB) by alternative non homologous end-joining (NHEJ), leading to increased repair infidelity that introduces genomic changes into DNA and drives disease progression. This cycle of DNA damage and misrepair is driven by increased endogenous reactive oxygen species (ROS) production. Treatment of transgenic mice with N-acetyl cysteine results in an up to 30-50% decrease in ROS, DNA damage and concomitant NHEJ misrepair activity. Finally, we find that increased ROS is produced through activation of RAC because specific inhibition of RAC significantly diminishes ROS activity in these mice. RAC is downstream of RAS and is activated by RAS signaling. RAC is also an essential component of NADPH oxidase that produces superoxide radicals. Results with RAC inhibition and antioxidant treatment suggest that combined therapy that may diminish the activities that drive disease progression. Our data serve as a model system to further study the role of activated RAS/MAP kinase pathways and increased ROS in propagating genomic instability and disease progression and could also lead to identification of downstream molecules for therapeutic targeting in an effort to stabilize the genome and halt disease progression in acute myelord leukemia.

Key words: genomic instability, DNA damage, acute myeloid leukemia

Resumen ***Inestabilidad genética como área potencial de intervención terapéutica***. La inestabilidad genómica es una de las propiedades que caracterizan a las enfermedades malignas mieloides y a los síndromes pre-leucémicos que predisponen a dichas leucemias. Nuestros resultados, en un modelo experimental en ratón transgénico para estudiar la progresión de la enfermedad mieloide, demuestran que la inestabilidad genómica en estas enfermedades dependería de una combinación entre daños constitutivos del ADN acoplados a una alteración en los mecanismos de reparación de las rupturas en la doble cadena (DSB). El mecanismo normal de reparación sería reemplazado por un mecanismo alternativo de unión de extremos no homólogos [non homologous end-joining (NHEJ)], y el conjunto de estos eventos llevaría a un aumento de la ineficiencia en la reparación de errores que finalmente resultaría en cambios en el genoma que facilitarían la progresión maligna de la enfermedad. Este ciclo de daños en el ADN, junto con fallas en su reparación, está asociado con un aumento en la producción de especies reactivas del oxígeno (ROS). El tratamiento de los ratones transgénicos con N-acetilcisteína resulta en una disminución del 30 al 50% de ROS, así como también en una importante reducción del daño en el ADN y en la concomitante atípica actividad de reparación NHEJ. Finalmente, también demostramos que el aumento de ROS es inducido a través de la activación de RAC, dado que se observó que la inhibición específica de RAC es capaz de inhibir la actividad de ROS en estos ratones. RAC se ubica río abajo de RAS y es activada a través de la señalización por RAS. RAC es también un componente esencial de la NADPH oxidasa, que produce radicales superóxido. Nuestros resultados con la inhibición de RAC y con el tratamiento anti-oxidante sugieren que una terapia combinada podría disminuir las actividades que conducen a la progresión de las enfermedades malignas mieloides. Nuestros datos pueden servir como modelo para profundizar el estudio del papel de la activación de las vías RAS/MAPK y del aumento de ROS en la propagación de la inestabilidad genómica y en la progresión maligna. Asimismo podrían llevar a la identificación de otras moléculas río abajo con potencial utilidad como nuevos blancos terapéuticos, en un esfuerzo para estabilizar el genoma y detener la progresión de la leucemia mieloide aguda.

Palabras clave: inestabilidad genómica, daño del ADN, leucemia mieloide aguda

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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 [1,2]. Although the mechanisms remain elusive, it is widely recognized that genomic instability accentuate the acquisition of molecular abnormalities (the mutator phenotype theory)[1-3]. In normal, non malignant cells there are pathways that maintain genomic integrity by repairing DNA damage without causing deletions or mutations[4]. 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[5], whereas NHEJ is an error-prone repair pathway. NHEJ has been extensively characterized in rodent cells (reviewed in[6]), identifying a pathway in which the sub-unit 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 Ku70/86 heterodimer[7-9]. The targeted free ends are subsequently ligated by DNA ligase IV in conjunction with XRCC4[10]. However, although NHEJ can repair breaks in an error-free manner, it can also introduce errors in the form of small DNA deletions of less than 30bp during repair[11]. Such errors, generated at an increased frequency, could contribute to the accumulation of mutations (these refer broadly to genomic alterations) in these cells[12,13]. We previously found that myeloid malignancies were characterized by significantly increased misrepair frequencies, compared with normal human myeloid progenitors (CD34+) cells. Importantly, the misrepair was characterized by large plasmid deletions (>250 bp) in our in vitro assays[12,13].

One scenario for the acquisition of genomic instability would be increased exposure to DNA damaging agents that lead to DSB, in combination with an inability to repair this DNA damage correctly. Coincidence of both increased DNA damage and ineffective repair could create the environment for the acquisition of genetic alterations, which is a hallmark of tumor progression. We previously reported significantly increased error-prone NHEJ in preleukemic syndromes and a variety of myeloid malignancies[12]. Furthermore, we demonstrated that these cells harbor constitutive DNA damage[13]. We postulated that increased NHEJ misrepair may be a response to the increased generation of DNA damage in myeloid malignancies, which could lead to a cycle of genomic instability that introduces further genetic changes into DNA[12-16]. Here, we have tested this hypothesis using a mouse model for MDS disease progression. We show below that there is increased DNA damage that is accompanied by an increase in the frequency of NHEJ repair errors. Furthermore, we find that this DNA damage is directly related in part to increased ROS production that can be reversed with antioxidant treatment in a RAC1-dependant manner.

We examined whether increased genomic instability, as measured by DNA damage and repair in a transgenic mouse model for myeloid disease progression, involving over expression of mutant *NRAS* and *BCL2* (Fig. 1). We assayed for DNA damage in myeloid progenitor cells (sca1+) from *NRAS* and *BCL2* single transgenic mice, using an established immunofluorescence-based assay for single stranded DNA (ss-DNA) damage, dependent on BrdU incorporation[17]. This assay relies on the fact that anti-BrdU antibodies can only detect BrdU incorporated

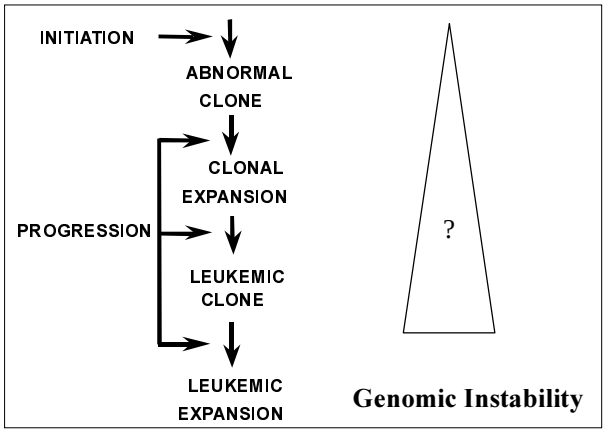

Fig. 1.– Model of leukemia progression. Preleukemic disease is initiated by one or more genetic events in a BM CD34+ progenitor cell. Expansion of this abnormal clone occurs. Disease progression involves additional genetic alterations. Transgenic *NRAS* mice have a mild phenotype; a small increase in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

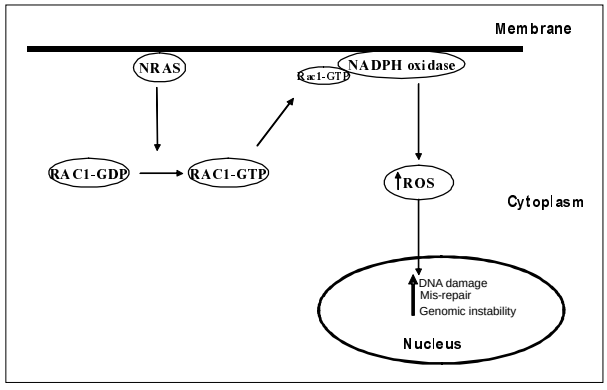

Fig. 2.– NRAS signaling leads to RAC1 activation and NADPH oxidase mediated production of ROS. Increased ROS leads to genomic instability.

into DNA when it is in single-stranded form. Upon examination of BrdU-incorporated chromatin fibers in myeloid progenitor cells (sca1+) from both *NRAS* and *BCL2* mice, we saw that *NRAS* and to a lesser extent *BCL2* demonstrate an increase in the frequency of constitutive DNA damage, compared with normal mice. Examination of sca1+ cells of *NRAS+BCL2* double transgenic mice showed that this damage increases significantly, compared with both the single transgenics and normal (FVBN) mice (Fig. 2).

To determine whether this DNA damage is a substrate for DSB repair, we performed co-immunofluorescence analysis with the variant histone γH2AX, a key protein in the repair of DSB[19]. Importantly, we find that this constitutive damage on chromatin fibers co-localizes with γH2AX. 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* vs *NRAS+BCL2* transgenic mice, to confirm the increased DNA damage and repair demonstrated 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 performed we demonstrated that the mean number of γH2AX foci per cell from normal FVBN spleen and BM was 2±2, whereas, cells from *BCL2* and *NRAS* transgenic mice gave 4±2 and 10±2 γH2AX foci, respectively. Importantly, spleen and BM cells from double transgenic mice gave an increased number of γH2AX foci per cell, above that for both normal and single transgenic mice, of 18±2 foci.

To determine whether the error-prone NHEJ pathway participates in the repair of the DSB that we have detected, we performed co-immunostaining of these regions of DNA damage with antibodies to protein components from the NHEJ repair pathway on chromatin fibers. We find that DNA damage co-localizes with the NHEJ protein Ku86. Furthermore, we also found that γH2AX co-localizes with Ku86. 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 demonstrated. We have previously shown that myeloid leukemia cells contain increased repair infidelity of the NHEJ repair pathway for DSB[12-14]. Therefore, we determined whether NHEJ repair fidelity was altered in sca1+ cells from the *NRAS* and *BCL2* transgenic mice, using an *in vitro* plasmid end-joining assay in cell free extracts. Nuclear extracts were prepared from BM and spleen cells and used in a *lac*Z plasmid reactivation assay; this colony assay allowed quantitation of correctly repaired colonies (blue) vs incorrectly repaired (white) colonies[20]. Bone marrow sca1+ cells from single transgenic mice (*NRAS, BCL2*) had an increased misrepair frequency, 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 to 400 bp) compared with FVBN cells (<30 bp).

Previous studies have shown that cell lines transduced with activating *HRAS* mutants demonstrate increased production of reactive oxygen species (ROS)[21]. 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[4, 22]. We, therefore, tested whether *NRAS* also causes increased ROS production in *NRAS/BCL2* transgenic mice, by staining cells with the fluorescent probe dihydrorhodamine-123 (DHR123). This compound is oxidized to the fluorophore rhodamine-123 by ROS with absorption/emission maxima of 507/529nm and was thus detected by flow cytometric analysis[23]. The integrity of the assay is demonstrated via the increased ROS production in myeloid progenitor cells (FDCP1 cell line) following H_2O_2 treatment; where H_2O_2 is a well established producer of ROS[24, 25]. We find that, as expected, H_2O_2 produces an increase in ROS above baseline levels. BM MNC from *NRAS* mice show increased ROS, compared with FVBN mice. Further increases in ROS above that exhibited by *NRAS* mice are seen in the *NRAS+BCL2* double transgenic. 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, B220+) compartment.

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 anti-oxidants, such as N-acetyl cysteine (NAC)[26]. 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 NAC. (40 mM; Sigma, St Louis, MO) in their drinking water for a period of 7 weeks to the end of gestation, according to the protocols of Reliene *et al*[27], and offspring were examined for DNA damage and repair. This same intake of NAC was shown to reduce DNA adduct formation in rats exposed to genotoxic agents [26]. The control group received acidic (pH 2.8) drinking water. We examined gH2AX foci formation in NAC treated and control untreated animals. We find a 30-50% reduction in the frequency and staining intensity of γH2AX foci in NAC treated animals, compared with untreated controls. These data indicate that some of the DNA damage caused by *NRAS* and *NRAS+BCL2 in vivo* is dependent on ROS production. We next examined NHEJ misrepair activity in NAC treated transgenic and control mice. We find no significant de-

crease in NHEJ misrepair activity following NAC treatment. However, BM MNC from double transgenic *NRAS + BCL2* mice treated with NAC mice showed a small but reproducible decrease in the frequency of NHEJ misrepair. Interestingly, however, the size of plasmid deletions examined from plasmid reactivation experiments showed a significant decrease in size, following NAC treatment. Therefore, both *in vivo* DNA damage and NHEJ misrepair activity caused by *NRAS* and *NRAS+BCL2* appear in part dependent on the production of ROS. However, this benefit did not translate into improved survival.

We next examined the underlying mechanisms responsible for production of ROS in *NRAS+BCL2* mice. Within the context of myeloid dysregulation we have previously demonstrated the role of RAC-mediated signaling pathways[28]. In addition, increased ROS may be produced by RAS signaling through RAC, a downstream target of RAS activation[29]. Consistent with the flow cytometry data, increased levels of RAC1-activty were observed within the Mac-1+ compartment as compared to B220+. 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 and incubated with the highly selective cell-permeable RAC1 inhibitor (NSC23766), followed by ROS measurement[30]. We find that the RAC1 inhibitor has a marginal effect on ROS levels in Mac-1+ cells from FVBN mice and reduced levels are observed in the B220+ cells of *RAS+BCL-2* mice. However, the RAC1 inhibitor has significant and titrable effect on the ROS activity in Mac-1+ sorted *NRAS+BCL-2* cells. This data is confirmed with RAC1 GTP-pull-down assays from *NRAS+BCL2* transgenic mice. While RAC1 activity is not significantly changed in B220+ cells following exposure to the RAC1 inhibitor, Mac-1+ cells show a dramatic decrease in RAC1-GTP in the presence of NSC23766.

References

1. Loeb L. Cancer cells exhibit a mutator phenotype. *Adv Canc Res* 1998; 72: 25-55.
2. Loeb LA. A mutator phenotype in cancer. *Cancer Res* 2001; 61: 3230-9.
3. Loeb LA. Mutator phenotype may be required for multistep carcinogenesis. *Cancer Research* 1991; 51: 3075-9.
4. Mills KD, Ferguson DO, Alt FW. The role of DNA breaks in genomic instability and tumorigenesis. *Immunol Rev* 2003; 194: 77-95.
5. Khanna KK, Jackson SP. DNA double-strand breaks: signaling, repair and the cancer connection. *Nat Genet* 2001; 27: 247-54.
6. Zdzienicka MZ. Mammalian mutants defective in the response to ionizing radiation-induced DNA damage. *Mutat Res* 1995; 336: 203-13.
7. Falzon M, Fewell JW, Kuff EL. EBP-80, a transcription factor closely resembling the human autoantigen Ku, recognizes single- to double-strand transitions in DNA. *J Biol Chem* 1993; 268: 10546-52.
8. Gottlieb TM, Jackson SP. The DNA-dependent protein kinase: requirement for DNA ends and association with Ku antigen. *Cell* 1993; 72: 131-42.
9. Mimori T, Hardin JA. Mechanism of interaction between Ku protein and DNA. *J Biol Chem* 1986; 261: 10375-9.
10. Grawunder U, Wilm M, Wu X, et al. Activity of DNA ligase IV stimulated by complex formation with XRCC4 protein in mammalian cells. *Nature* 1997; 388: 492-5.
11. Critchlow SE, Jackson SP. DNA end-joining: from yeast to man. *Trends Biochem Sci* 1998; 23: 394-8.
12. Gaymes TJ, Mufti GJ, Rassool FV. Myeloid leukemias have increased activity of the nonhomologous end-joining path-way and concomitant DNA misrepair that is dependent on the Ku70/86 heterodimer. *Cancer Res* 2002; 62: 2791-7.
13. Brady N, Gaymes TJ, Cheung M, Mufti GJ, Rassool FV. Increased error-prone NHEJ activity in myeloid leukemias is associated with DNA damage at sites that recruit key nonhomologous end-joining proteins. *Cancer Res* 2003; 63: 1798-05.
14. Gaymes TJ, North PS, Brady N, Hickson ID, Mufti GJ, Rassool FV. Increased error-prone non homologous DNA end-joining-a proposed mechanism of chromosomal instability in Bloom's syndrome. *Oncogene* 2002; 21: 2525-33.
15. Rassool FV. DNA double strand breaks (DSB) and nonhomologous end joining (NHEJ) pathways in human leukemia. *Cancer Lett* 2003; 193: 1-9.
16. Rassool FV. Genetic rearrangements beget genomic instability. *Blood* 2004; 104: 3424-5.
17. Raderschall E, Golub EI, Haaf T. Nuclear foci of mammalian recombination proteins are located at single-stranded DNA regions formed after DNA damage. *Proc Natl Acad Sci USA* 1999; 96: 1921-6.
18. Rassool FV, North PS, Mufti GJ, Hickson ID. Constitutive DNA damage is linked to DNA replication abnormalities in Bloom's syndrome cells. *Oncogene* 2003; 22: 8749-57.
19. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem* 1998; 273: 5858-68.
20. North P, Ganesh A, Thacker J. The rejoining of double-strand breaks in DNA by human cell extracts. *Nucleic Acids Res* 1990;18: 6205-10.
21. Irani K, Xia Y, Zweier JL, et al. Mitogenic signaling mediated by oxidants in Ras-transformed fibroblasts. *Science* 1997; 275: 1649-52.
22. Karanjawala ZE, Murphy N, Hinton DR, Hsieh CL, Lieber MR. Oxygen metabolism causes chromosome breaks and is associated with the neuronal apoptosis observed in DNA double-strand break repair mutants. *Curr Biol* 2002; 12: 397-402.
23. Khwaja A, Tatton L. Resistance to the cytotoxic effects of tumor necrosis factor alpha can be overcome by inhibition of a FADD/caspase-dependent signaling pathway. *J Biol Chem* 1999; 274: 36817-23.
24. Boveris A, Chance B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem J* 1973; 134: 707-16.
25. Mouzannar R, Miric SJ, Wiggins RC, Konat GW. Hydrogen peroxide induces rapid digestion of oligodendrocyte chromatin into high molecular weight fragments. *Neurochem Int* 2001; 38: 9-15.
26. Balansky R, Izzotti A, Scatolini L, D'Agostini F, De Flora

S. Induction by carcinogens and chemoprevention by N-acetylcysteine of adducts to mitochondrial DNA in rat organs. *Cancer Res* 1996; 56: 1642-7.
27. Reliene R, Fischer E, Schiestl RH. Effect of N-acetyl cysteine on oxidative DNA damage and the frequency of DNA deletions in atm-deficient mice. *Cancer Res* 2004; 64: 5148-53.
28. Omidvar N, Pearn L, Burnett AK, Darley RL. Ral is both necessary and sufficient for the inhibition of myeloid differentiation mediated by Ras. *Mol Cell Biol* 2006; 26: 3966-75.
29. Irani K, Goldschmidt-Clermont PJ. Ras, superoxide and signal transduction. *Biochem Pharmacol* 1998; 55: 1339-46.
30. Akbar H, Cancelas J, Williams DA, Zheng J, Zheng Y. Rational design and applications of a Rac GTPase-specific small molecule inhibitor. *Methods Enzymol* 2006; 406: 554-65.

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The Moving Finger writes, and having writ,
Moves on: nor all your Piety nor Wit
Shall lure it back to cancel half a Line,
Nor all your Tears wash out a Word of it.

El Dedo Movedizo escribe, y habiendo escrito,
Sigue adelante: ni su Piedad ni su Sabiduría
Podrán atraerlo de vuelta para cancelar media Línea,
Ni podrán sus Lágrimas borrar una sola Palabra.

Edward FitzGerald (1809-1883)

The Rubaiyat of Omar Khayyam