BCL-2 and Mutant NRAS Interact Physically and Functionally in a Mouse Model of Progressive Myelodysplasia

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Abstract

Myelodysplastic syndromes (MDS) are clonal stem cell hematologic disorders that evolve to acute myeloid leukemia (AML) and thus model multistep leukemogenesis. Activating RAS mutations observed in hematopoietic malignancies are the most frequent molecular abnormality in MDS and are linked to poor survival and transformation to AML. The effect of mutant RAS is structure and cell context dependent. The family of RAS proteins includes HRAS, KRAS, and NRAS, whose oncogenic potential can be activated by point mutations in the GTP-binding domains around codons 12 and 61. The biological consequences of expressing mutant NRASD12 in vitro in human primary CD34+ cells include impairment of proliferation, increased cell cycle time, and increased apoptosis. Likewise, in murine hematopoietic FDC-P1 cells, mutant NRASD12 induces proliferation and reduced growth factor dependence in vitro. When MDS transforms into AML, the increased apoptosis seen in low-risk refractory anemia MDS patients’ CD34+ cells is reduced and an increase of anti-apoptotic signals is observed. Cell biology and iron kinetic studies have shown that proliferation of the stem cell is linked with intramedullary cell death, which explains the ineffective hematopoiesis and often hypercellular marrow. As BCL-2 has been observed to be up-regulated in AML patients (16, 17) and blocks differentiation of myeloid progenitors (18), it may be considered as a potential candidate progression gene of MDS transformation. Indeed, mouse models involving members of the BCL-2 family such as the proapoptotic BID-deficient mice develop an MDS-chronic myelomonocytic leukemia–like disease (19).

We hypothesized that the combined expression of BCL-2 and mutant NRAS would establish a mouse model of progression to leukemia. Thus, herein, we describe two distinguishable high-penetration in vivo models of MDS and AML. Furthermore, by balance between cell proliferation, apoptosis, and differentiation, with dysregulation of these genes, leading to an expansion of normal stem cells. Abnormalities in certain stem cell genes are needed to dysregulate cell proliferation and also to bypass controls that would normally trigger apoptosis, differentiation, or senescence (4). Combinations of oncogenes that transform mouse cells and transgenic models of various malignancies have been described (5, 6). However, few studies have analyzed the progression of genetic events leading to the expansion of the leukemic clone. Approximately 30% of myelodysplastic syndrome (MDS) patients evolve to acute myelogenous leukemia (AML). This progressive and indolent evolution suggests the acquisition of several gene abnormalities.

Mutations of NRAS can occur in patients at risk of leukemia transformation after chemotherapy and/or radiotherapy (7, 8). We and others have shown that NRAS mutations at codon 12 is the most frequent molecular abnormality in MDS and is linked to poor survival and transformation to AML (9–11). The effect of mutant NRAS is structure and cell context dependent. The family of RAS proteins includes HRAS, KRAS, and NRAS, whose oncogenic potential can be activated by point mutations in the GTP-binding domains around codons 12 and 61. The biological consequences of expressing mutant NRASD12 in vitro in human primary CD34+ cells include the inhibition of proliferation, increased cell cycle time, and increased apoptosis (12). Likewise, in murine hematopoietic FDC-P1 cells, mutant NRASD12 induces proliferation and reduced growth factor dependence in vitro (13). When MDS transforms into AML, the increased apoptosis seen in low-risk refractory anemia MDS patients’ CD34+ cells is reduced and an increase of anti-apoptotic signals is observed. Cell biology and iron kinetic studies have shown that proliferation of the stem cell is linked with intramedullary cell death (14), which explains the ineffective hematopoiesis and often hypercellular marrow (15). As BCL-2 has been observed to be up-regulated in AML patients (16, 17) and blocks differentiation of myeloid progenitors (18), it may be considered as a potential candidate progression gene of MDS transformation. Indeed, mouse models involving members of the BCL-2 family such as the proapoptotic BID-deficient mice develop an MDS-chronic myelomonocytic leukemia–like disease (19).

We hypothesized that the combined expression of BCL-2 and mutant NRAS would establish a mouse model of progression to leukemia. Thus, herein, we describe two distinguishable high-penetration in vivo models of MDS and AML. Furthermore, by
taking advantage of the conditional tetracycline-inducible system, we show that the triple transgenic MMTVtTA/TBCL-2/NRASD12 develop an MDS with increased blasts and that BCL-2 expression is necessary for both initiating this neoplasm and for fatal disease progression. We provide mechanistic evidence of the colocalization and complexing of BCL-2 with active-RAS, which we also see in MDS/AML patients, and show different signaling pathways between the two models.

Materials and Methods

Transgenic mice. Generations of transgenic mice are described in detail in Supplementary Experimental Procedures. All procedures complied with European or national regulations.

Tissue and cell preparation. Blood was obtained from anesthetized animals (with isoflurane) by venipuncture of retro-orbital venus plexus into EDTA tubes. Differential blood counts were obtained using an automated hematology analyzer running veterinary software (Hematvet 850, CDC Technologies). Bone marrow was obtained by flushing long bones with HBSS followed by filtering through a nylon mesh. Blood and bone marrow smears were prepared according to standard hematologic techniques. Bone marrow smears were examined by cytologists from the Cardiff School of Medicine, King's College Hospital and Hôpital Saint-Louis. The tissue sections were examined by the Head of Histopathology of Hôpital Saint-Louis, and classified according to the Bethesda proposal (20) where blast equivalents are designated as "immature forms/ blasts," which for the purposes of convenience are referred to as blasts herein. Percentage blasts were determined from the bone marrow smears by counting 100 to 200 cells. Lin−, Sca-1− (marker of early progenitor), and Mac-1− (integrin equivalent of human CD11b) fractions were separated using an AutoMacs separator (Miltenyi). The lineage depletion kit contained a mixture of biotinylated antibodies CD5 (T-cell antigen), CD45R (lymphocyte antigen), Mac-1, Gr-1(Ly5-6G) (granulo-macrophagic differentiation antigens), and Ter119 (early erythroid antigen). Cell sorting for Lin−/Sca-1−/c-Kit+ bone marrow cells were undertaken using anti-Sca-1 antibody conjugated with FITC and anti–c-Kit antibody conjugated with allophycocyanin (Becton Dickinson). Cell sorting was undertaken using an LSR (Becton Dickinson). Livers and spleens were fixed overnight in buffered formalin and embedded in paraffin and sectioned by the Moffit Hospital, University of California San Francisco or Hôpital Saint-Louis, IUH Histopathology departments.

Immunophenotyping by flow cytometry. Mice were anesthetized with isoflurane and peripheral blood samples were obtained by orbital bleeds using EDTA as anticoagulant. Twenty-five microtiter aliquots of peripheral blood cells were immunophenotyped with antibodies conjugated with either phycoerythrin or FITC. Bone marrow and spleen cells were analyzed using Sca-1, c-Kit, Mac-1, Gr-1, B220 (B cell), and CD3 (T cell) (Becton Dickinson). When required, cells were permeabilized with "Fix and Perm" (Caltag) and labeled with an antihuman BCL-2 antibody conjugated to FITC (Becton Dickinson). Cells were analyzed on a FACS Calibration cytometer and analysis was undertaken using the Cell Quest software (Becton Dickinson). At least three independent analysis of each genotype were investigated.

Figure 1. Triple transgenic MMTVtTA/TBCL-2/NRASD12 mice develop high-risk MDS, whereas MRP8/BCL-2/NRASD12 transgenic mice show AML progression. A, H&E-stained bone marrow smears and organ sections showing increased blast cells [low-power (LP) magnification, ×40] with liver and spleen infiltration in the triple transgenic mice. At high power (HP, ×120), representative normal neutrophils are arrowed (N in black), as well as dysplastic cells (D arrowed in black) and quantified blast cells (B arrowed in white). MRP8/BCL-2/NRASD12 spleen sections show a reduction and dispersion of the white pulp and increase of the red pulp due to an increase in extramedullary hematopoeisis, also seen in the liver. As previously reported, single MRP8/BCL-2 or MRP8/NRASD12 transgenic mice show no leukemic phenotype in liver and spleen sections during the time of observation of up to 6 mo (24, 49). Nevertheless, the spleens of MRP8/NRASD12 mice were injected i.v. in tail veins of immunocompromised RAG1-deficient mice, which are B-cell and T-cell deficient (22). Mice were followed for survival and some cells were harvested, and blast counts were determined and transferred into secondary or tertiary recipients. Disease was confirmed by blast cell counts, and expression of the H2-9 phenotype of FVB/N mice whereas recipient B6 mice have H2-9 haploptypes, with genotyping of peripheral blood DNA or confirmation of BCL-2 expression in Lin− cells by immunofluorescence.

Immunofluorescence and confocal microscopy. Mononuclear cells of murine or human bone marrow were examined as described in Supplementary Experimental Procedures.

RAS activation assays and Western blotting. Procedures were as described in Supplementary Experimental Procedures.

Statistical analysis. Described in Supplementary Experimental Procedures.

Results

Transgenic mice coexpressing BCL-2 and NRASD12 give rise to MDS/AML-like diseases in a promoter context–dependent manner. We sought to test the hypothesis that the development of AML is a multistep process by creating two different models coexpressing BCL-2 (23) with mutant NRASD12 (24): (a) a tetracycline-regulatable model where BCL-2 expression is conditionally induced by the MMTVtTA transactivator in which the addition of the tetracycline analogue doxycycline down-regulates the triple transgenic mice. At high power (HP, ×120), representative normal neutrophils are arrowed (N in black), as well as dysplastic cells (D arrowed in black) and quantified blast cells (B arrowed in white). MRP8/BCL-2/NRASD12 spleen sections show a reduction and dispersion of the white pulp and increase of the red pulp due to an increase in extramedullary hematopoeisis, also seen in the liver. As previously reported, single MRP8/BCL-2 or MRP8/NRASD12 transgenic mice show no leukemic phenotype in liver and spleen sections during the time of observation of up to 6 mo (24, 49). Nevertheless, the spleens of MRP8/NRASD12 mice are enlarged; this is due to reactive proliferation of cells as a result of their susceptibility to develop infections and not to malignancy. This phenomenon has been previously described as a susceptibility of these mice to develop hyperkeratotic lesions, which become secondarily infected with fecal flora, resulting in the proliferation of myeloid cells (24). Significantly increased blast cell populations were observed in bone marrow samples of single MRP8/BCL-2 versus FVB/N mice (P < 0.005; n = 5) and MRP8/BCL-2/NRASD12 versus FVB/N mice (P < 0.00005; n = 4). B, histograms of blood counts demonstrating increased WBC, reduced platelet counts with uncharged RBC, and increased organ/body weight ratios. C, bone marrow immunophenotyping: The MMTVtTA promoter drives disease with expansion toward the myeloid compartments (Mac-1/Gr-1), showing in MMTVtTA/TBCL-2/NRASD12 increased R1 to R2 ratio (bottom), thus depicting a blast cell expansion.

Representative of three independent experiments. D, TUNEL staining of paraffin-embedded liver sections; low-power (×200) and high-power (×600) magnifications show significantly increased apoptosis in MMTVtTA/TBCL-2/NRASD12 versus controls. Three fields were counted in each of the two mice in each group.

Colonies assays. Colony assays were performed using the Methocult medium as recommended by the manufacturer (Stem Cell Technologies) and is described in Supplementary Experimental Procedures.

Apoposis assays. Apoptosis was assessed by in situ detection of fragmented DNA, using the terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) assay on 5-μm paraffin-embedded liver sections (21). Quantitative data on tissue sections were assessed blindly by two pathologists (A.J. and C.L.) on an Olympus Provis AX-70 microscope, with wide-field eyepiece number 26.5, providing a field size of 0.344 mm² at ×400 magnification. Cell counts were performed on three different fields per section, and expressed as the mean number of cells per field at ×400 magnification using the Olympus SIS software system.

Transplantation of bone marrow and spleen cells. Bone marrow was isolated from both tibias and femurs and spleen cells were harvested from 6- to 10-week-old MRP8/BCL-2/NRASD12 mice as described above, pooled and divided (10⁶ nucleated cell aliquots per recipient) for i.v. injections into 12 irradiated FVB/N mice. Six- to 8-week-old FVB/N mice were prepared for transplantation by cesium irradiation totaling 10 Gy, divided into two doses 3 to 4 h apart. Successful transfer of the transgene-positive cells was confirmed by PCR. Spleen cells (10⁷, 10⁵, or 10³) from MMTVtTA/TBCL-2/NRASD12 mice were injected i.v. in tail veins of immunocompromised RAG1-deficient mice, which are B-cell and T-cell deficient (22). Mice were followed for survival and some cells were harvested, and blast counts were determined and transferred into secondary or tertiary recipients. Disease was confirmed by blast cell counts, and expression of the H2-9 phenotype of FVB/N mice whereas recipient BALB/c mice have H2-9 haploptypes, with genotyping of peripheral blood DNA or confirmation of BCL-2 expression in Lin− cells by immunofluorescence.


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granulated, nondystrophic/pathologic myeloblasts with the capacity to mature was noted (12 ± 2%; n = 3; Supplementary Fig. S1A), and a survival similar to the wild-type FVB/N mice (data not shown). An MMTVtTA/TBCL-2 line was crossed with the MRP8NRASD12MMTVtTA mice (24) to generate transgenic MMTVtTA/TBCL-2/NRASD12 mice. The MRP8NRASD12 mice had dysplastic differentiated bone marrows with normal blood counts (Fig. 1A; Supplementary Fig. S1C). MMTVtTA/TBCL-2/NRASD12 mice have increased WBC counts (P < 0.05), reduced platelet counts (P < 0.001), and enlarged spleens and livers (P < 0.001 and P < 0.05 compared with FVB/N and MMTVtTA controls; Fig. 1B). Morphologically, neutrophils of MMTVtTA/TBCL-2/NRASD12 mice are dysplastic, with segmented nuclei; some may be vacuolated, with less nuclear appendages and a finer chromatin (Fig. 1A). The excess of blasts in the MMTVtTA/TBCL-2/NRASD12 bone marrow averages 15 ± 3% (n = 3; Fig. 1A). Compared with wild-type FVB/N, bone marrow immunophenotyping showed an increase in Sca-1+ cells (data not shown), consistent with the increased marrow blasts, and an increase in Mac-1+/Gr-1low (RI) subpopulations (Fig. 1C, top and bottom) consistent with the myeloid left shift. The liver sections did not show aggressive invasiveness although there were some infiltrations of myeloblasts. Spleen sections showed reduction and dispersion of the white pulp and increase of the red pulp due to an increase in extramedullary hematopoiesis (Fig. 1A). These results indicate that the disease of MMTVtTA/TBCL-2/NRASD12 mice resembles that of human high-risk MDS [FAB (25) or WHO (26) classifications]; the thrombocytopoiesis and neutrophil dysplasia do not meet the Bethesda classification criteria for myeloid leukemia but are consistent with MDS (20).

For the generation of the constitutive BCL-2 mice, MRP8BCL-2 (23) mice were crossed with MRP8NRASD12 (24) mice. Compared with the MMTVtTA/TBCL-2/NRASD12 model, a significantly higher percentage of bone marrow blasts is noted (89 ± 4%, n = 4) with aggressive invasiveness in the livers and spleens (Fig. 1A). Whereas, as previously described (23, 24), MRP8NRASD12 (n = 43) and MRP8BCL-2 (n = 65) transgenic mice have survival patterns resembling wild-type FVB/N mice (n = 76); MRP8BCL-2/NRASD12 mice (n = 31) have a significantly poorer survival with 50% (16 of 31) dying within 3 to 4 months (P < 0.0001; Supplementary Fig. S1B). Mortality of these mice is due to a myeloid neoplasm that progressed from MDS to myeloid leukemia without maturation. Before death, blood counts show quantitative abnormalities, among which progressive decrease of platelets is the most indicative (mean 2-fold; P < 0.001; Supplementary Fig. S1C). With peripheral cytopenia and increased marrow blasts, the disease of the MRP8BCL-2/NRASD12 mice may be classified as human AML-like (FAB and WHO classifications; refs. 25, 26) or murine myeloid leukemia without maturation (Balthesda classification; ref. 20). Immunophenotyping of hematopoietic tissues substantiate the leukemic pathology of the MRP8BCL-2/NRASD12 mice. Compared with wild-type mice, the bone marrow Sca-1+ population is increased 3.5-fold in the MRP8BCL-2/NRASD12 mice, and 1.5-fold and 2.5-fold in the MRP8NRASD12 and MRP8BCL-2 mice, respectively (data not shown). This is also consistent with the observed increase in blast cells in the bone marrow of the single and double MRP8 transgenic mice, with the MRP8BCL-2 bone marrows revealing a proportion of myeloid blast cells (18 ± 5%, n = 5; Fig. 1A). Significantly increased bone marrow populations were positive for the myeloid markers, Mac-1 and Gr-1, in the MRP8NRASD12 and MRP8BCL-2/NRASD12 mice compared with FVB/N wild-type mice, with a highly significant increase in the number of Mac-1+/Gr-1low myeloid cells (R1) in the double transgenic mice, indicating a left shift in maturation of the Mac-1+/Gr-1low subpopulation (Supplementary Fig. S1D, top and bottom), which correlated well with increased bone marrow blast infiltration (Fig. 1A).

As MDS is characterized by increased apoptosis of hematopoietic cells, tissues from mouse liver sections were analyzed by a TUNEL assay to further substantiate the observed MDS morphologic features. As expected, increased apoptosis was detected when MDS features were dominant, such as in single MRP8NRASD12 and MMTVtTA/TBCL-2/NRASD12 mice; however, no apoptotic cells were noted when blast infiltration was abundant as in the leukemic MRP8BCL-2/NRASD12 mice (Fig. 1D). Morphologically, the positive staining cells were hematopoietic.

Consistent with the expanded Sca-1+ population in both models (data not shown), the leukemic stem cell compartmentalization of these primitive cells were next confirmed by confocal microscopy for exogenous hBCL-2 alongside the Sca-1 and c-Kit markers in Lin−/Sca-1−/c-Kit+ enriched MMTVtTA/TBCL-2/NRASD12 bone marrow cells (see Fig. 2A for a representative cell). Additionally, Lin− sorted samples from both MDS-like and AML-like mice showed a significantly raised Sca-1−/c-Kit− population versus control mice (Fig. 2B, top and bottom). A significant increase in myeloid colony numbers was observed in MMTVtTA/TBCL-2/NRASD12 and MRP8BCL-2/NRASD12 compared with MMTVtTA and FVB/N controls (P < 0.05 and P < 0.005, respectively; Fig. 2C).

The myeloid neoplastic disease of the MMTVtTA/TBCL-2/NRASD12 was serially transplantable into RAG1 mice as evidenced by blast counts from bone marrow smears (Supplementary Fig. S2A). Similarly, the MRP8BCL-2/NRASD12 model was transplantable into RAG1 and lethally irradiated mice as evidenced by pathology (Supplementary Fig. S2B) and immunophenotyping (Supplementary Fig. S2C). As both MMTVtTA/TBCL-2/NRASD12 and MRP8BCL-2/NRASD12 mice were serially transplantable in immunocompromised RAG1 mice up to four passages (data not shown), this suggests that in both models the disease originated in a leukemic stem cell. This is reinforced by the observation that unlike the Lin+ populations, Lin− cells from diseased MDS-like and AML-like mice were transplantable into RAG1 or lethally irradiated FVB/N mice (Supplementary Fig. S2A and data not shown). No significant difference between the mice was observed in the other cell populations; the percentage of lymphoid cells in the leukemic MRP8BCL-2/NRASD12 mice was similar to single MRP8BCL-2 mice (data not shown).

Thus, the coexpression of NRASD12 with BCL-2 in the myeloid cells of these mice results in two models of leukemogenesis: one model of high-risk MDS (MMTVtTA/TBCL-2/NRASD12) and one model of AML (MRP8BCL-2/NRASD12).

BCL-2 is a rate-limiting step. As the two models described suggest that at least two steps (expression of BCL-2 and mutated NRAS) are required for leukemogenesis, we investigated whether extinction of one of these steps reversed the neoplastic disease. To test this, we took advantage of the inducible MMTVtTA/TBCL-2/NRASD12 model (tetracycline-off, in which the addition of doxycycline down-regulates transgene expression). When doxycycline is withdrawn, MMTVtTA/TBCL-2/NRASD12 mice die within 2 months (five of five), compared with no death in six mice with doxycycline treatment, and with death of the majority of mice untreated with doxycycline within 6 months (n = 18; Fig. 3A, right). When we administered doxycycline at week 3 and continued treatment...
until week 12, a correction of the disease symptoms, such as thrombocytopenia ($P < 0.001$; Fig. 3B) and hyperleukocytosis ($P < 0.005$; Fig. 3C), and recovery of normal survival were achieved (Fig. 3A, right). Expression of hBCL-2 and the development of the myeloid neoplastic disease could be switched off and on repeatedly by withdrawing or administering doxycycline, respectively, for a maximum of three times over the follow-up period of 22 months with WBC reverting to that of $\text{MRP8[NRASD12]}$ mice (Fig. 3C).

However, although the bone marrow of doxycycline-treated $\text{MMTVtTA/TBCL-2/NRASD12}$ mice shows a correction of disease with increased survival and a slight but reproducible reduction of bone marrow blast cells ($15 \pm 3\%$ dropping to $8 \pm 3\%$; $n = 3$; Fig. 3A, left), immunophenotyping suggested persistence of bone marrow blast cells with Mac-1$^+/\text{Gr-1low}$ cell remaining as high as the untreated mice (data not shown), whereas the Sca-1$^+$ (as well as the c-Kit$^+$) population remained unchanged (Fig. 3D; Supplementary Fig. S3A), indicative of some remaining disease and possibly other steps. Likewise, organs remained infiltrated with immature myeloid cells and spleens were still enlarged (Supplementary Fig. S3B) similar in size to $\text{MMTVtTA/TBCL-2/NRASD12}$ spleens yet distinct from the enlarged reactive spleens of $\text{MRP8[NRASD12]}$ mice, which are not infiltrated by blasts (Fig. 1A, top; previously described in ref. 24). These persistent abnormalities, despite reduced hBCL-2 expression, suggest that the coexpression of NRASD12 and BCL-2 has driven additional lesions that are irreversible. Nevertheless, the corrections of most of the hematopoietic variables in the absence of hBCL-2 correlated well with a normal life expectancy during a follow-up period of up to 600 days (Fig. 3A, right).

**BCL-2-RAS-GTP protein complexes occur in the Sca-1$^+$ compartment of diseased mice.** Activating point mutations of RAS lock RAS in their GTP-bound active state. As the combination of the BCL-2 and NRASD12 transgenes is able to initiate the development of the malignancy in these mice, we asked whether RAS activity was sustained in $\text{MRP8[BCL-2/NRASD12]}$ and $\text{MMTVtTA/TBCL-2/NRASD12}$ Sca-1$^+$ cells coexpressing NRASD12 and BCL-2 and asked whether a common molecular mechanism exists. Within the Sca-1$^+$ and Mac-1$^+$ compartments of the spleen cells of $\text{MRP8[BCL-2/NRASD12]}$ and $\text{MMTVtTA/TBCL-2/NRASD12}$ mice, increased levels of GTP-bound RAS are present, a finding also noted in the Mac-1$^+$ sorted populations of the single BCL-2 and NRASD12 mice. Expression of hBCL-2 was confirmed using a species-specific antihuman BCL-2 antibody (Fig. 4A). Interestingly, in this population, only the phosphorylated form of BCL-2 protein (27) was predominantly observed. RAS activity was assessed using an activation-sensitive pull-down assay, whereby RAS in its GTP-bound configuration is detected via the RAS-binding domain of its downstream effector RAF1 (28, 29). Increased RAS activity was observed in the Sca-1$^+$ and Mac-1$^+$ cells of $\text{MMTVtTA/TBCL-2/}$
NRASD12 mice, which persisted in the absence of hBCL-2 expression after doxycycline treatment (Fig. 4A, top). This may account for the persistence of disease detected by tissue invasion and sustained increase of Sca-1+ cells (Fig. 3A, left, and data not shown). Within the Sca-1+ compartment of the MRP8[BCL-2/NRASD12] spleen cells, increased levels of GTP-bound RAS are also noted (Fig. 4A, middle). Interestingly, hBCL-2 probing of the active RAS pull-down assay proteins revealed the presence of BCL-2 in the RAS complex (BCL-2:RAS-GTP) in untreated MMTVtTA/TBCL-2/NRASD12 mice (Fig. 4A, top). These experiments were also performed with MMTVtTA/TBCL-2/NRASD12 mice treated with doxycycline for 3 and 12 weeks in which BCL-2 expression is significantly extinguished. The complexes were correlated by a time-dependent decrease of BCL-2. The presence of the BCL-2:RAS-GTP complex was also observed in MRP8[BCL-2/NRASD12] mice (Fig. 4A, middle). As described above,
Figure 4. Increased RAS activity in Sca-1+ cells of MRP8[BCL-2/NRASD12] and MMTVtTA/TBCL-2/NRASD12 mice due to BCL-2:RAS-GTP complexing and colocalization of the complex. A, top, Sca-1+ and Mac-1+ sorted spleen cells from MMTVtTA/TBCL-2/NRASD12 mice without (Untreated) or with doxycycline treatment for 3 and 12 wk; middle, wild-type FVB/N, MRP8[BCL-2], MRP8[NRASD12], and MRP8[BCL-2/NRASD12] mice were assessed for total RAS expression by Western blotting and total BCL-2 expression by reprobing the blots with a human specific anti–BCL-2 antibody (hBCL-2). Active RAS-GTP levels were assessed via a sensitive RAF1-RBD pull-down assay followed by Western blotting with an anti-RAS antibody. Blots were reprobed with anti-actin antibody to assess protein loading. Results shown are representative of three independent experiments. Bottom, RAF1-RBD pull-down assay of FVB/N, untreated, and doxycycline-treated diseased MMTVtTA/TBCL-2/NRASD12 mice, demonstrating persistence of mBCL-2:RAS-GTP complex irrespective of hBCL-2 levels. B, immunofluorescence microscopy of MMTVtTA/TBCL-2/NRASD12 mice bone marrow cells shows loss of human BCL-2 (hBCL-2) in the presence of doxycycline for 12 wk. DAPI, 4',6-diamidino-2-phenylindole. C, confocal microscopy showing subcellular localization of BCL-2:RAS complex. hBCL-2, RAS, and mitochondria (Tom20) or plasma membrane (WGA) stained cells of unsorted bone marrow cells in MMTVtTA/TBCL-2/NRASD12 and MRP8[BCL-2/NRASD12] mice showing mitochondrial localization of hBCL-2 and RAS in MRP8[BCL-2/NRASD12] mice and reduction of mitochondrial colocalization in MMTVtTA/TBCL-2/NRASD12 mice, with plasma membrane colocalization of MMTVtTA/TBCL-2/NRASD12 and a reduction of colocalization with WGA in the MRP8[BCL-2/NRASD12] mice (representative of at least three independent experiments). D, colocalization of BCL-2 and RAS when NRAS is mutated in human samples. Immunofluorescence microscopy of bone marrow cells from patient 1 with a secondary MDS/AML presenting with mutant NRAS, and from patient 2 with secondary MDS/AML with wild-type NRAS, and from patient 2 with secondary MDS/AML with normal human bone marrow. Colocalization of hBCL-2 and RAS is observed only in the presence of mutant NRAS.
after doxycycline treatment, although lethality is rescued, myeloblast infiltration remains. Mechanistically, RAS activity remained high in the Mac-1+ spleen cells even when hBCL-2 is switched off (Fig. 4A, top). We therefore hypothesized the recruitment of endogenous mBCL-2 within the complex. RAF1-RBD pull-down assay of FVB/N, untreated, and doxycycline-treated diseased MMTVtTA/TBCL-2/NRASD12 mice showed persistence of mBCL-2:RAS complex irrespective of hBCL-2 levels (Fig. 4A, bottom). Although in vitro interaction between BCL-2 and RAS has been previously reported in Jurkat cells (30) and R-RAS, a RAS-related protein detected with BCL-2 using a yeast two-hybrid system (31), this is the first presentation of data linking the unique presence of BCL-2 in an NRAS-activated complex to the development of a malignant disease. The persistence of activated RAS despite significant decrease of BCL-2 illustrates the initiation of irreversible steps.

Using confocal and immunofluorescence microscopy, we substantiate that hBCL-2 and RAS are colocalized in MMTVtTA/TBCL-2/NRASD12 and MRP8/BCL-2/NRASD12 cells from granulocyte-macrophage colony-forming unit (CFU-GM) colonies as well as unseparated and Lin- separated bone marrow cell populations (Fig. 4B and C; Supplementary Fig. S4A and B). Importantly, we confirm the controlled extinction of the colocalized proteins via immunofluorescence microscopy of MMTVtTA/TBCL-2/NRASD12 bone marrow cells in the absence of lethality when hBCL-2 expression is turned off with the administration of doxycycline and the persistence of the colocalization of RAS and mouse BCL-2 (Fig. 4B).

To identify the subcellular localization of the BCL-2:RAS complex in the MMTVtTA/TBCL-2/NRASD12 and MRP8/BCL-2/NRASD12 mice, triple staining of RAS, BCL-2, and mitochondria or plasma membrane was undertaken (Fig. 4B and C) and colocalizations were quantified (Supplementary Fig. S4C). Despite >50% of the cells of the MMTVtTA/TBCL-2/NRASD12 with colocalization of RAS and hBCL-2, only a minority of the cells (~10%) colocalize in the mitochondria, whereas in the MRP8/BCL-2/NRASD12 mice, most of the bone marrow cells, which were RAS and hBCL-2 positive, were found to colocalize in the mitochondria (Fig. 4C and Supplementary Fig. S4C). Bone marrow Lin- isolated cells confirmed these findings with 50% of BCL-2 and RAS colocalizing, 12% of hBCL-2 colocalizing with mitochondria, and nearly no RAS colocalizing in the mitochondria (1%) of the MMTVtTA/TBCL-2/NRASD12 mice, whereas 30% of MRP8/BCL-2/NRASD12 cells with hBCL-2 and RAS colocalized, 20% of the hBCL-2 colocalized with mitochondria, and practically all of the RAS in the complex appeared to localize in the mitochondria (25%; Supplementary Fig. S4B and C). In contrast, with the plasma membrane probe wheat germ agglutinin (WGA), we observed hBCL-2 and RAS colocalizations in the MMTVtTA/TBCL-2/NRASD12 mice of around 40% and a reduction in the MRP8/BCL-2/NRASD12 mice at ~7% (Supplementary Fig. S4C).

To confirm the relevance of this observation in human MDS/AML, we analyzed the coexpression of BCL-2 and RAS in the mononuclear cells of the bone marrow of MDS/AML patients (n = 6) with known RAS mutation status (32). Interestingly, colocalizations of BCL-2 and RAS in MDS/AML patient samples but not in a normal individual was also observed (Fig. 4D). The colocalization of BCL-2 and RAS appears to be correlated with the mutated status of RAS (n = 2 with RAS mutations and n = 3 wild-type). Although these preliminary results require confirmation on a larger cohort of MDS/AML patients, such an association in the pathogenesis of MDS/AML patients has not been previously described.

to further dissect the mechanisms involved, we interrogated the mitogen-activated protein kinase and AKT signaling pathways in Mac-1 and Sca-1 sorted bone marrow cells from diseased animals versus healthy controls (Fig. 5). We used previously reported optimized conditions by others and ourselves (33, 34). In the MMTVtTA/TBCL-2/NRASD12 cells, the proapoptotic phenotype corresponds with the reduced levels of phosphorylated-AKT, with increased phosphorylated extracellular signal-regulated kinase (ERK) compared with the MMTVtTA in the Sca-1+ compartment (Fig. 5A). Furthermore, phosphorylated ERK is up-regulated in the MRP8/NRASD12 single transgenic line as well as the Mac-1+ compartment of the MRP8/BCL-2/NRASD12 (Fig. 5B). In the Sca-1+ compartment, where we postulate that BCL-2 is dominant based on its apoptosis profile, phosphorylated AKT is up-regulated, consistent with the antiapoptotic characteristics of cells from this line (Fig. 5B).

Discussion

We have created two murine models of initiation and progression of human MDS/AML, using two candidate genes, mutant NRAS and BCL-2. Both have previously been identified by us and others as risk factors for AML in MDS patients (9, 10, 16, 17). The MMTVtTA/TBCL-2/NRASD12 mouse model represents human MDS, whereas the constitutive MRP8/BCL-2/NRASD12 model is closer to AML. This distinction is also reflected by the presence of apoptosis (as observed in the human low-risk MDS stage of the disease) seen in the hematopoietic tissues in the livers of MMTVtTA/TBCL-2/NRASD12 mice and its decreased presence or absence in the MRP8/BCL-2/NRASD12 mice (as in the human high risk MDS and AML), which is also reflected by the signaling profiles of these mice. Furthermore, the subcellular localization of the two oncogenes when complexed appears to be consistent with their apoptotic characteristics. In the MDS-like mice, which like single...
transgenic MRP8NRASD12 mice are proapoptotic, the majority of the RAS and BCL-2 doubly stained cells did not colocalize in the mitochondria, but localized to the plasma membrane, where active RAS is normally located, whereas in the AML-like disease RAS and BCL-2 colocalize more so in the mitochondria, where BCL-2 is normally found and is consistent with the antiapoptotic properties of BCL-2. Interestingly, in Madin-Darby canine kidney cells and Jurkat T-cells, protein kinase C (PKC) agonists have been found to induce mutant KRAS to dissociate from the plasma membrane where activated RAS proteins are normally found and to translocate to the mitochondria where it associates with BCL-XL to stimulate apoptosis (35). Although mutant KRAS was found to associate with BCL-2, this interaction was found to be insensitive to PKC agonists. The difference in our findings in the AML-like mice, which appear to complex NRASD12 and BCL-2 in the mitochondria to give rise to reduced apoptosis, may be due to the different cell types and context. The coexpression of these two genes is necessary for the disease to appear as both of the single transgenic mice, NRASD12 or BCL-2, have mild phenotypes and normal survival. The stem cell features of these diseases are defined by their ability to repopulate immunodeficient Rag1 or lethally irradiated mice. This is confirmed by the finding that the disease of the MMTVtTA/TBCL-2/NRASD12 and MRP8[BCL-2/NRASD12] mice can be successfully transplanted into Rag1 or lethally irradiated FVB/N mice using Lin− isolated cells in the absence of disease or repopulation from the Lin+ fraction. Furthermore, there are increased primitive Sca-1−, Mac-1+/Gr-1low, and Lin+ / Sca-1+/c-Kit− bone marrow cell populations in both mice, and an increase in progenitors compared with wild-type mice, indicating diseases of primitive cells. Indeed, hBCL-2 expression was observed in bone marrow of Lin− / Sca-1−/c-Kit− sorted cells from MMTVtTA/ TBCL-2/NRASD12 mice, showing that the mouse mammary tumor virus–long terminal repeat (MMTV-LTR) can drive expression to this primitive compartment. Furthermore, the MRP8[BCL-2/ NRASD12] and the MMTVtTA/TBCL-2/NRASD12 mice also present with enlarged infiltrated spleens and liver characteristic of malignant cells. Sca-1-directed BCL-2 overexpression in the aorta-gonad-mesonephros region, wherein the mouse embryo first hematopoietic stem cells are generated, and also results in increased numbers of Sca-1− and c-Kit+ cells (36). We have shown that expression of BCL-2 in human myeloid progenitors blocks differentiation (18). In the single transgenic BCL-2 mouse, we have noted some immature Sca-1− cells and marrow blasts. NRASD12 mice show myeloid dysplastic features but no expansion of the Sca-1+ population and no blast infiltration with well-differentiated cells. Few other abnormalities are seen and, thus, both the single NRASD12 mouse and the single BCL-2 mouse have a normal phenotype and survival. We surmise that in the MRP8-targeted myeloid progenitor, the combined effects of constitutively active NRASD12 and expression of active BCL-2 lead to an expansion of immature myeloid cells at the expense of differentiation. Therefore, it would appear that MMTV-LTR and MRP8, which do not usually target hematopoietic stem cells, in the context of the BCL-2RASGTP complex give rise to leukemic stem cells. However, the target cells may be different depending on where BCL-2 is expressed; either the mutant NRAS is dominant and the cells fail to differentiate and die by apoptosis, giving rise to a human MDS-like disease (MMTVtTA/TBCL-2/NRASD12), or antiapoptotic BCL-2 is dominant, leading to a block in differentiation and a reduction in apoptosis, and blast cells accumulate, giving a human AML-like disease (MRP8[BCL-2/NRASD12]).

The activation-sensitive GST-RAF1-RBD immunoprecipitation assays allowing quantification of active RAS levels showed that, indeed, activated RAS in the Sca-1− compartment is only detected in the MRP8[BCL-2/NRASD12] and the MMTVtTA/TBCL-2/ NRASD12 transgenic mice and not in the single NRASD12, BCL-2, or normal FVB-N mice, which suggests that the activity of this complex in a primitive compartment may be contributing to disease progression. The elevated total RAS and RAS-GTP levels in the diseased transgenic mice might be due to BCL-2–mediated increased survival of cells that express high levels of NRASD12, which might otherwise undergo cell cycle arrest or senescence. Interestingly, only the phosphorylated form of BCL-2 is found in this cell population where RAS is activated. Investigation of the molecular mechanisms involved within the increased primitive Sca-1− cell population revealed the first in vivo identification of the BCL-2RAS-GTP complex in the context of the disease, suggesting that the association between these oncoproteins may mediate an underlying mechanism. Extinction of one of these genes, BCL-2, rescues the lethality of the disease. A preliminary study of patient samples lead to the discovery of a similar colocalization in the bone marrow of patients with MDS/AML harboring a mutated NRAS. Although the role of phosphorylated BCL-2 is controversial (37), these observations provide further insight into the mechanisms by which association of activated RAS with BCL-2 is context dependent in the transduction of proapoptotic versus antiapoptotic signaling during hematopoietic development. This is consistent with the signaling profile where ERK is increased with decreased AKT in the RAS-mediated proapoptotic MDS-like mice and AKT is increased in the BCL-2–mediated antiapoptotic AML-like disease.

Numerous in vivo murine RAS models have been reported presenting with different diseases. Models established with either viral Ha-ras or with Ha-ras driven by a Moloney murine leukemia virus LTR as well as transgenic mice established with a codon 61 mutant NRAS (38–41) present with proliferative lymphoid diseases. Furthermore, transplantation of bone marrow cells retrovirally transduced with NRASD12 (42) or mutant KRAS conditionally expressed in Mx1+ cells result in myeloproliferative disorders (34, 43). The last two models are particularly elegant in that despite the physiologic levels of mutant KRAS driven by its own promoter, alone a lethal disease ensues rapidly due to the Mx1 targeting a different progenitor to the MRP8-driven NRASD12 used in our present study. Tetracycline-regulatable mutant NRASV12 mice crossed with VAVtTA mice results in a reversible systemic mastocytosis in 2 to 4 months (44). Thus, distinct phenotypes may be obtained, implying that the in vitro structure/context–dependent effect of the different RAS proteins is also reflected in vivo. NRASD12 is one of the most frequent mutations found in MDS or AML. Both MDS and AML affect the myeloid stem cells and therefore justifies the use of a myeloid promoter, such as MRP8. As the MRP8 promoter is a target of C/EBPα in human CD34+ cells, the concomitant-driven expression of both transgenes in the primitive Sca-1− compartment is to be expected (45). The models established in this study closely mimic the human MDS/AML. MRP8 appears to be turned on in a subset of granulocyte/macrophage progenitors (46), and it would appear that granulocyte/macrophage progenitors can become leukemic stem cells (47–49). Whereas these two models provide insights into the leukemogenesis of the patients through the required cooperation of these two disease candidate genes to expand the primitive compartment to form leukemic stem cells, the regulatable MMTVtTA/TBCL-2/ NRASD12 model shows that the expression of BCL-2 is necessary
for both initiation and progression of malignancy; BCL-2 accelerates the development of neoplasia in these models as it does in other mouse models of acute promyelocytic and chronic myelogenous leukemia (47, 48). However, close analysis of the hematopoietic compartments shows the irreversibility of some variables such as the persistence of elevated Sca-1+ population in the peripheral blood, blasts in the bone marrow, and splenomegaly with infiltration of the liver, and thus indicates that although extinction of BCL-2 expression rescues from lethality, some degree of disease still exists, consistent with the multistep mechanisms involved in a progressive disease like MDS. In our model, we hypothesize that mutant NRAS can recruit mBCL-2 to remain active. We provide evidence from the pull-down and confocal assays to show that mouse BCL-2 binds with active RAS and colocalizes with RAS. The complex association of BCL-2 with active RAS may lead to irreversible steps if mBCL-2 and NRAS are left to cooperate until overt leukemia appears.

We have shown that at least two candidate oncogenes (BCL-2 and mutant NRAS) can cooperate to give rise to malignant disease with a penetrance of around 80% and a latency period of 3 to 6 months. The demonstration that one of them (BCL-2) is a rate-limiting step may therefore be a target for future therapy in this group of diseases. This study validates an in vivo strategy to create molecularly defined multistep models with specific gene abnormalities that cause a disease with hallmarks of MDS and that transform to AML. These models will be important to decipher the BCL-2-RAS-GTP complex and the contribution of other genetic abnormalities that may accelerate disease progression, and to decipher novel therapeutic approaches.

Acknowledgments

Received 1/16/2007; revised 9/6/2007; accepted 10/15/2007.

Grant support: Fulbright Commission, Welsh Bone Marrow Transplant Fund, UK Leukaemia Research Fund, Kay Kendall Research Fund, Eli Lilly International Foundation, ANR, Foundation of Cancer Research, European Leukemia Network, NIH, and Institut National de la Sante et de la Recherche Medicale; and NIH (D. Felsher).

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Scott Kogan is a Scholar of the Leukemia and Lymphoma Society.

We thank Michael Bishop for hosting the early part of this study; Jack Whittaker for reviewing the hematologic slides; Christopher Marshall (Institute for Cancer Research, London, United Kingdom) for providing the pGEX-2T-RAS-RHGS construct; Fabien Zasadzinski, Thi Hai Phan, Macarena Robledo, Sacha Muzelak, Liebheid Mutter, Katerina Pokorna, Aurore Cleret, and Lucie Da Silva for technical assistance; members of the Institut Universitaire d’Hématologie IFR165 specifically the Département d’Exper- imentation de la Léukémie; Bernard Bourdin from the photography laboratory, Christelie Doliger of the Imagery Department of the confocal microscope, who are supported by grants from the Conseil Regional d’Ile-de-France, and the Ministère de la Recherche. The authors have no conflicting financial interest.

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Announcements

MEETING OF THE RADIATION RESEARCH SOCIETY

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22-24, 1953. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on "The Effects of Radiation on Aqueous Solutions," which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is "Physical Measurements for Radiobiology" and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray's lecture is sponsored by the Iowa Branch of the American Cancer Society. Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelmann, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

ERRATUM

The following correction should be made in the article by Beck and Valentine, "The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration," November, 1952, page 891; substitute for the last paragraph:

The data in Table 8 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to O₂ consumption, it is seen that the amount of glucose "cleavage products" exceeds the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and is exceeded by the glucose utilized by 16 per cent in CLL. If the assumption is made that, in this respect, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if M = +0.27 and L = -0.16 and the normal differential is 65 per cent M and 35 per cent L, then

\[ 0.65 (+0.27) + 0.35 (-0.16) = +0.12 \]

a figure identical to the observed +0.12 for normal leukocytes.
BCL-2 and Mutant NRAS Interact Physically and Functionally in a Mouse Model of Progressive Myelodysplasia

Nader Omidvar, Scott Kogan, Stephanie Beurlet, et al.


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