Abstract

Hypersensitivity to chemo- and radiotherapy employed during cancer treatment complicates patient management. Identifying mutations in genes that compromise tissue recovery would rationalize treatment and may spare hypersensitive patients undue tissue damage. Genes that govern stem cell homeostasis, survival, and progenitor cell maintenance are of particular interest in this regard. We used wild-type and c-myb knock-out mice as model systems to explore stem and progenitor cell numbers and sensitivity to cytotoxic damage in two radiosensitive tissue compartments, the bone marrow and colon. Because c-myb null mice are not viable, we used c-myb heterozygous mice to test for defects in stem-progenitor cell pool recovery following γ-radiation and 5-fluorouracil treatment, showing that c-myb+/− mice are hypersensitive to both agents. While apoptosis is comparable in mutant and wild-type mice following radiation exposure, the crypt beds of c-myb+/− mice are markedly depleted of proliferating cells. Extrapolating from these data, we speculate that acute responses to cytotoxic damage in some patients may also be attributed to compromised c-myb function.

Introduction

Bone marrow (BM) and the gastrointestinal tract are continuously renewing tissues containing highly ordered stem and progenitor cell compartments with tightly controlled proliferation, differentiation, and apoptosis throughout life. Despite insights gained from analysis of mutant mice with specific genetic lesions, the molecular control of homeostasis in BM, and the gastrointestinal tract in particular, is poorly understood. Analysis of c-myb heterozygous mutant (c-myb+/−) mice has confirmed that this gene is essential for establishing definitive hematopoiesis (1) and colonic crypt formation (2). Whether c-myb contributes to the maintenance of these two compartments once established in adult animals is unclear.

C-myb has been studied extensively in the hematopoietic system and blood cell malignancies. It is highly expressed in immature cells of most lineages and declines as cells differentiate (3). However, the role of c-myb in regulating hematopoiesis is complex because it seems to be employed and required at multiple differentiation steps (4-6). The overexpression of c-myb, or expression of activated forms of Myb that transform hematopoietic cells, implicates c-myb in hematopoietic malignancies (7-9). Conversely, very few mature adult-like blood cells are generated by the c-myb+/− fetal liver (1), and c-myb+/− mice die at the critical developmental checkpoint when fetal liver erythropoiesis is established (1, 6).

C-myb is also essential for colon development and its overexpression modifies normal colon biology. Basic expression data show that rat, mouse, and human colons have levels of c-myb mRNA similar to, or exceeding, that observed in thymus and BM (10-13). Colon cancer cells from humans (14-16) and rodents (11, 17, 18) frequently overexpress c-myb compared with normal colon mucosa and differentiation of colon cancer cells results in down-regulation of c-myb in a fashion comparable to that observed with c-myb in differentiating hematopoietic cells (10). Importantly, c-Myb increases during colon (19) and esophageal adenocarcinoma progression (20). In colon cancer, this increase is important because the level of c-myb expression has prognostic significance for patient survival (21).

Coloncic crypts are exquisite structures that generate a vast and continuous epithelial surface allowing efficient water and ion reabsorption. As with other rapidly renewing tissues like the skin and hematopoietic system, the architectural features and function of each colonic crypt is maintained by a fine balance of high proliferation through the provision of progenitor cells that arise from a single steady-state stem cell at the crypt base; cell differentiation into columnar, goblet, and endocrine cells as they migrate away from the base; and apoptosis into the crypt lumen near or at the top of the crypt. C-Myb expression is strongest at the crypt base and decreases toward the middle of the crypt where it partitions to be expressed weakly in differentiated columnar cells (10, 12). Understanding the regulation of colonic crypt homeostasis is key to unraveling colon hyperproliferative disorders, initiation of colon cancer, and recovery of colon integrity following tissue damage. We have reported that one functional allele of c-myb is required for fetal crypt formation in the mouse.
Although c-myb haploinsufficiency has little or no demonstrable effect under steady-state conditions, we reasoned that cytotoxic challenge might reveal a stress response role for this gene. Both colonic crypts and BM undergo rapid renewal and their stem and progenitor cell populations are extraordinarily sensitive to cytotoxic insult. In the case of BM, we find c-myb expression in the most immature stem cell population, and c-myb expression is at its strongest in the bottom third of the colonic crypt. Thus, such immature cells in these tissues were the focus of our study. We employed ionizing radiation and 5-fluorouracil (5-FU) to stress these two highly proliferative compartments. Ionizing radiation kills both stem cells and progenitor cells, whereas a single 5-FU treatment preferentially kills progenitor cells. Such cytotoxicity necessitates the recruitment of new cells to allow tissue regeneration. Under these conditions, c-myb+/- mice show significant impairment in BM and colonic crypt recovery compared with wild-type controls. Crypt beds are depleted of cells in the c-myb heterozygous mice leading to their progressive disintegration. These observations suggest that c-myb is required for stem and/or progenitor cell renewal in BM and colonic crypts.

**Results**

**Crypt Length and Induced Apoptosis Are Identical in c-myb+/- and Wild-Type Mice Following Ionizing Radiation Treatment**

c-myb-/- fetal colon fails to form normal crypts (2) and the distribution of apoptosis is markedly different in these mutant transplants (see Discussion). Assessment of the specific consequences of c-myb loss in adult colon has not been investigated. Thus, although the hematopoietic (1, 6) and gastrointestinal tract (data presented below) compartments of adult c-myb+/- mice seem essentially normal, we speculated that differences in the regenerative ability of c-myb+/- hematopoietic stem cells and colonic crypt cells might be revealed when these mice were challenged by irradiation or chemotherapeutic drugs.

Wild-type and c-myb+/- mice at 4 hours, 2, 3, and 4 days post-lethal irradiation were examined. Irradiation resulted in a precipitous decline in BM cellularity from 21.0 ± 1.2 (c-myb+/-) and 19.8 ± 2.0 (c-myb+/-) nucleated cells per femur, respectively, at 4 hours to 1.7 ± 0.3 (c-myb+/-) and 1.4 ± 0.2 (c-myb+/-) nucleated cells per femur at day 4 post-irradiation (data not shown). Although abnormal morphologic changes were evident by day 3 in c-myb+/- colons, no significant differences in cell number or apoptosis (Fig. 1A–G) were observed until day 4.

**c-myb Heterozygous Crypts Disintegrate at Day 4 Post-Irradiation**

Sections from c-myb+/- mice at day 4 were not amenable to morphometric analysis because the crypt architecture was disrupted and frequently hypocellular (Fig. 2A). By day 4, the cycle of radiation-induced apoptosis was minimal as judged by the absence of pyknotic bodies (see Fig. 1E and G) indicative of apoptosis (22-24). The disintegration of the normal architecture (Fig. 2A) that is essentially restored in the wild-type mice (Fig. 2B) is shown for four heterozygous mice. Higher magnification of an additional two heterozygous colon sections (Fig. 2C) reveals either empty crypt beds depleted of cells or poorly defined crypt structures compared with wild-type colons (Fig. 2D). This is the location in which stem and progenitor cells normally reside, suggesting that these populations of cells have been selectively deleted in the c-myb+/- crypts.

**Similar Apoptosis but Markedly Different Proliferation in Wild-Type and c-myb+/- Colons**

Detecting apoptotic bodies in normal crypts is problematic because most apoptosis occur at the luminal surface such that...
dead cells are transported away by peristalsis or engulfed by macrophages. However, cells in the early stages of apoptosis can be identified using antibodies directed against activated caspase-3. Figure 3A and B demonstrate that apoptosis occurs in both c-myb heterozygous and wild-type crypts in a similar fashion. Intense staining for pro-apoptotic Bak was also equally evident in both wild-type and mutant colons (not shown). Therefore, it seems that enhanced apoptosis at either the crypt base or luminal surface is not primarily responsible for the radiosensitivity of the c-myb+/− colons.

Attempts to identify mitotic figures in the recovering crypts of c-myb+/− mice at day 4 suggested that cell division was not restored compared with the wild-type colons. To verify this, we examined a marker of cell proliferation frequently used to examine gastrointestinal tract cell turn over. PCNA expression serves as an informative indicator of proliferation in colonic crypts where its location is normally restricted to the bottom third of the crypt (12). c-Myb expression is also most intense in this region, persisting in differentiated columnar cells in mice and humans (10, 12). In c-myb+/− colon 4 days post-irradiation, some crypts showed essentially no PCNA staining at the crypt base, perhaps due to crypt base hypocellularity. PCNA expression was mostly evident distal to the crypt base (Fig. 3C and D), suggesting that in c-myb+/− colon, the stem cell compartment was not replacing progenitor cells as they differentiate and eventually apoptose.

To characterize the apparent differences in proliferation, colonic crypts from five wild-type and five heterozygous mice at day 4 post-irradiation were examined in terms of the crypt position(s) where PCNA staining occurred. Conceptually, adult colonic crypts have one steady-state stem cell at the base located at position 0. With division, daughter cells occupy positions moving up from the base starting at position 1. The actual number of functional stem cells is thought to increase following radiation (25). Figure 4A shows that PCNA staining in heterozygous crypts is very heterogeneous unlike wild-type crypts (Fig. 4B). PCNA-positive nuclei in 20 individual crypts per mouse were scored using the central cell at the crypt base as the starting position 0. Figure 4B shows the highly significant difference (P > 0.001, ANOVA) for the frequency of PCNA-positive nuclei in heterozygous mice at positions 0, 1, 2, and 3. On average, wild-type crypts showed 2 times or greater number of positive nuclei at the crypt base. This is most evident at position 0 where 75% of cells in this position (15 of 20) show PCNA staining compared with 30% (6 of 20) in heterozygote crypts. It was also of interest that cells in position 1 showed significantly less PCNA staining compared with positions 0 (P > 0.02) and 2 (P > 0.002). Distinct differences at position 1 compared with positions 0 and 2 have been observed in other instances. For example, in mid-colonic crypts at 24 hours post-irradiation, apoptosis is less at position 1 compared with position 0 or 2 (26). This cell position may represent the first and most primitive “progenitor” cell. Collectively, these data suggest that c-Myb is required to maintain proliferation at the crypt base and, because stem and progenitor cells reside in this region, we further suggest that c-Myb is required for progenitor cell expansion.

**5-FU Treatment Reveals a Defect in c-myb Heterozygous BM Stem Cells**

Ionizing radiation ablates the vast majority of stem and progenitor cells at the doses employed in this study, irreversibly compromising the BM stem cell reserve (17, 27). In comparison, 5-FU preferentially ablates proliferating progenitor cells and spares highly quiescent stem cells with long-term repopulating ability (LTRA; ref. 28). We have employed the high proliferation potential-colony forming cell (HPP-CFC) assay as a surrogate measure of the status of the hematopoietic stem cell compartment and the low proliferation potential-colony forming cell (LPP-CFC) assay as a measure of the committed progenitor cell pool (29). Comparison of the regenerating BM of 5-FU–treated mice revealed significant differences in the pattern of recovery for the incidence (Fig. 5C) and content (Fig. 5D) of high proliferation potential-colony forming cells (P = 0.005 and P = 0.001, respectively), but not low proliferation potential-colony forming cells, in c-myb+/− mice compared with wild-type controls. The incidence and femoral content of low proliferation potential-colony forming cell were essentially the same in both wild-type and c-myb

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**FIGURE 2.** Heterozygous mice show colonic crypt disintegration at day 4 post-irradiation. A, Low-power fields of four c-myb heterozygous distal colons at day 4 post-irradiation show aberrant morphology with abnormal levels of cellular debris within the lumen compared with similarly treated wild-type colons (B). C, High-power fields of additional two mice show that c-myb+/− crypts were disrupted and hypocellular (indicated by arrows) compared with crypts from two independent wild-type colons (D).
heterozygous BM, consistent with the view that c-myb haploinsufficiency blocks the early recovery or recruitment of stem cells under such stress conditions (Fig. 5A and B). Notably by day 5, the c-myb+/−/C0 BM high proliferation potential-colony forming cell numbers return to near normal levels.

5-FU Treatment Reveals a Defect in c-myb Heterozygous Colonic Crypt Recovery

Colonic crypt recovery was also significantly delayed in 5-FU–treated c-myb+/− mice compared with wild-type mice at days 1 and 5 posttreatment (Fig. 6A) (P = 0.02 and P = 0.033, respectively). Similarly, there were less apoptotic cells in the c-myb+/− crypts at day 1 (Fig. 6B) (P = 0.0005), essentially excluding a primary defect in the degree of apoptosis induction. As expected, mitosis was severely retarded in crypts of both genotypes until day 5 (Fig. 6C), suggesting that the difference in crypt length recovery is not due to a cell division defect per se in the heterozygous mice but a delay in replenishing progenitor cells. Indeed both wild-type and heterozygous crypt length exceeded the steady-state levels at day 5 although the heterozygotes recovery was delayed.

Collectively, these data suggest that c-myb haploinsufficiency results in a stem cell defect that impairs mouse BM and colonic crypt recovery following cytotoxic damage. Ionizing radiation leads to crypt disintegration, preventing the longer-term recovery (day 4) of c-myb+/− crypts. Moreover, analysis of BM and colonic crypt recovery following 5-FU suggests that the loss of one c-myb+/− allele impedes the replacement of transit amplifying progenitor cells that by definition are stem cell derived.

Discussion

The requirement for c-myb in the stem and progenitor cell populations of the fetal liver and BM, respectively, is most evident when c-myb knock-out and knockdown mutant mice...
are examined (1, 6). Because c-myb knock-out mice are not viable as adults, we employed wild-type and apparently normal c-myb heterozygous mice treated with agents that kill stem and progenitor cells to probe steady-state hematopoiesis. Under these conditions, adult c-myb−/− mice show a defect in stem cell recovery following 5-FU treatment.

At present, there are no ex vivo assays for colonic crypt stem and progenitor cell activity equivalent to the predictive colony forming assays used as a surrogate measure of hematopoietic stem cell activity in BM and fetal liver cells (29). However, documenting crypt cell numbers, mitosis, and apoptosis by morphometric assessment of crypts in vivo can be very informative in cases in which genetic defects influence these parameters. Both ionizing radiation and 5-FU treatment revealed significant differences in the recovery and survival of stem and progenitor cells in the colon of c-myb−/+ mice compared with wild-type controls. This was most evident when crypts were stained for proliferation antigen, PCNA, in which irradiated heterozygous crypts showed a substantial deficit in proliferation.

Therefore, the combined examination of colonic crypt and BM cells using in vivo and ex vivo assays in c-myb−/+ and wild-type mice has advanced our understanding of the role of c-myb in stem and progenitor cells, suggesting that diploid levels of c-myb are required for tissue recovery.

The defect in c-myb−/+ mouse colon and BM recovery is unlikely to be due solely to the absence of the c-Myb target gene bcl-2 or an increase in induced apoptosis. One report suggested that a Bel-2 deficiency led to colon and small intestinal disorganization (30), whereas others reported a normal gut phenotype in bcl-2−/− mice with elevated spontaneous apoptosis that was further increased following cytotoxic damage (24). In contrast, c-myb−/+ colons had normal steady-state crypt apoptosis and cell death that was either the same or lower following cytotoxic damage compared with wild-type colon. This was puzzling because previously we reported that apoptotic cells were more frequent in colon grafts from c-myb−/− fetuses (2). We now suggest that this difference between the knock-out and wild-type colon transplants is due to the inability of the knock-out grafts to extrude dead cells because of the lack of defined crypts with internal mucinous channels that exit into the lumen. In wild-type grafts, apoptotic bodies are not trapped and thus accumulate at the top of crypts and are evident because peristalsis does not operate in the graft tissue.

Some insights into the regulation of stem and progenitor cell homeostasis have been gained in which c-myb expression has been reduced. Blocking the characteristic c-myb mRNA induction observed when resting T cells are stimulated to exit G0-G1 (31-33) using antisense oligonucleotides suggests that the induction observed when resting T cells are stimulated to exit. Blocking the characteristic c-myb mRNA induction observed when resting T cells are stimulated to exit G0-G1 using antisense oligonucleotides suggests that a threshold level of c-myb is required for this process. This requirement may also be paralleled for quiescent BM and colonic crypt stem cells to reenter the cell cycle to give rise to progenitor cells.

A single 5-FU treatment did not seem to deplete proliferating progenitor cells to a greater extent in the c-myb−/+ mice as measured directly by BM colony forming assays. However, the high proliferation potential assay revealed a significant delay in recovery at day 1 but not the characteristic rebound of measurable stem cell activity at days 3 and 5 in BM of the heterozygous mice. These data suggest that the c-myb−/+ bone stem cell population is not inherently more sensitive to the antimitabolic effects of 5-FU, because this defect would be evident in the low proliferation potential population as well, but rather stem cells are slower to reenter into cycle, divide, and generate cell colonies ex vivo. This may be due to slower recruitment of cells with “stem cell”–like properties that emerge under stressed conditions, such as 5-FU treatment.

An alternative but not mutually exclusive view is that diploid levels of c-myb are required to maintain normal radioresistance and the loss of one copy sensitizes stem cells in crypts and perhaps BM (although this was not examined). The crypt data suggest that c-myb−/+ stem cells do not recover at the doses of radiation used in these studies. These questions will need to be addressed in an in vitro setting in which
epithelial and BM stem and progenitor cells are isolated from c-myb heterozygous mice. This will assist in avoiding the potential compounding problems that irradiation of tissue stroma and microvessels brings to in vivo experiments. In the case of the c-myb null fetal liver, a defect in the stromal production of stem cell factor (SCF) and the reduction of c-kit on the few evident hematopoietic cells (34) is likely to contribute to the overall deficit in high proliferation potential and low proliferation potential in the c-myb null embryo (35). Perhaps these two target genes are also in haploinsufficiency in the c-myb+/− BM such that this reduces recovery of stem cells following 5-FU treatment.

Collectively, these findings have implications that were not predicted. Extrapolating from the mouse c-myb+/− data it is tempting to suggest that in humans, impaired c-Myb function need not be evident under non-stressed conditions but may manifest severe BM and colon cytotoxicity when challenged during radio- and/or chemotherapy. Because there are groups of patients that respond in such a manner during cancer treatment, adjuvant therapy, and BM depletion before transplantation, investigating c-Myb function in these individuals should be considered.

Materials and Methods

Mice
c-myb+−/− mice have been described previously (1, 2) and were maintained on a C57Bl/6J background and housed in SPF Thoren racking system.

BM Colony Forming Assays
Colony-forming cells of low and high proliferation potential were assayed in a double layer nutrient agar culture system as previously described (36).

Radiation and 5-FU Treatments
A single dose of lethal whole body irradiation (13 Gy) was delivered at a rate of 67 cGy/min. Mice were sacrificed at 4 hours, 2, 3, and 4 days post-irradiation. 5-FU (David Bull Laboratories, Mulgrave, Australia) was made up to 15 mg/mL in saline, administered to mice (150 mg/kg, i.p.), and tissues harvested from euthanized mice were analyzed at days 1, 3, 5, and 7 administration.

Morphometric Analysis and Immunohistochemistry
Distal colon sections were fixed in 4% buffered paraformaldehyde overnight, embedded, sectioned, and H&E stained. Full crypts exposing a lumen and identifiable base were scored. Crypt cells per 40 to 50 longitudinal sections were scored and mitotic figures and apoptotic bodies enumerated based on established morphologic criteria (22-24). Sample groups were subjected to ANOVA using Origin software. Mouse anti-PCNA antibody, PC-10 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), was used at a 1:100 dilution to identify PCNA followed by goat anti-mouse horseradish peroxidase at 1:250 (Bio-Rad, Hercules, CA). Anti-activated caspase-3 (Promega, Madison, WI) was used at 1:500 and detected with goat anti-rabbit horseradish peroxidase at 1:250 (Bio-Rad). Each was developed with Pierce Metal enhanced detection reagent (Pierce, Rockford, IL).

FIGURE 5
Bone marrow from c-myb+−/− mice shows hypersensitivity to 5-FU. BM cells were plated under conditions that allow colony formation of cells with low proliferative potential (LPP) and high proliferative potential (HPP) per 25,000 plated cells (A and C) or per femur (B and D), in the c-myb+−/− mice compared with wild-type controls at days 1 to 5. The c-myb+−/− recovery of stem cells was significantly impaired compared with the wild-type bone marrow based on absolute numbers or per femur.
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