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A degradation-resistant c-Myb mutant cooperates with Bcl-2 in enhancing proliferative potential and survival of hematopoietic cells

Francesca Corradini^{1,2}, Rita Bussolari², Davide Cerioli¹, Maria Rosa Lidonnici¹, and Bruno Calabretta¹

¹Department of Microbiology and Immunology, Kimmel Cancer Center, Thomas Jefferson Medical College, Philadelphia, PA, USA

²Department of Biomedical Sciences, University of Modena e Reggio Emilia, Modena, Italy

Abstract

The c-myb gene is preferentially expressed in primitive hematopoietic cell and plays a central role in the control of cell proliferation, differentiation and survival by regulating the transcription of several genes implicated in these processes including the antiapoptotic Bcl-2.

We show here that, compared to wild-type c-Myb, overexpression of a degradation resistant c-Myb mutant [(358-452) c-Myb] enhances the clonogenic potential of hematopoietic progenitors as indicated by increased cytokine-dependent primary and secondary colony formation of Lin[−] Sca-1⁺ Kit⁺ mouse marrow cells.

Moreover, proliferation assays of IL-3 dependent myeloid precursor 32Dcl3 cells co-expressing Bcl-2 and c-Myb indicate that these cells continue to proliferate in the absence of IL-3 and this effect is more apparent in cells expressing the degradation resistant (358-452) c-Myb.

Interestingly, overexpression of (358-452) c-Myb is by itself sufficient to protect 32Dcl3 cells from apoptosis induced by IL-3 deprivation; moreover, these cells are also increased in number which most likely reflects the enhanced proliferative potential conferred by (358-452) c-Myb to apoptosis-resistant cells.

Keywords

oncogene cooperation; clonogenic potential; apoptosis resistance

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Correspondence: Francesca Corradini, Department of Biomedical Sciences, University of Modena e Reggio Emilia, Modena, Italy. E-mail: corraf@libero.it.

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INTRODUCTION

The c-Myb proto-oncogene is a DNA binding transcription factor involved in myeloid and lymphoid development [1, 2]. c-Myb is preferentially expressed by hematopoietic progenitor cells and its levels are downmodulated as cells terminally differentiate and withdraw from the cell cycle [3, 4]. The role of c-Myb in hematopoiesis is achieved through transcriptional regulation of genes important for proliferation, differentiation and survival (reviewed in *Wolff et al., 1996*) [5]. The c-Myb protein has a short half-life of ~30 minutes and undergoes proteasome-dependent degradation [6].

Truncations in the amino- and carboxy-termini lead to an increase of the transforming and transactivating activity of c-Myb. Indeed, c-Myb mutants lacking the aminoacid region 358-452 (which includes the C-terminal leucine zipper) were more stable than wild type c-Myb and induced increased primary and secondary colony formation in transduced hematopoietic cells [7].

One of the c-Myb target genes is Bcl-2, whose expression is important for the maintenance of early progenitor cells [8, 9, 10]. Bcl-2 is a member of the Bcl-2 family which regulates programmed cell death (reviewed in *Chao et al. 1998*) [11, 12, 13]. The Bcl-2 gene is associated with tumorigenesis in the B-cell lineage [14]. The co-expression of Bcl-2 and c-Myc has been shown to promote transformation of different mouse cell types (reviewed in *Adams and Cory, 2007*) [15]. Bissonnette et al. demonstrated that the cooperation between Bcl-2 and Myc in lymphomagenesis depends on the ability of Bcl-2 to inhibit Myc-induced apoptosis [16]. Constitutive c-Myb expression is associated with up-regulation of Bcl-2 mRNA and protein levels and protects transduced cells from apoptosis induced by growth factor deprivation [17].

To assess the transformation potential of a c-Myb mutant with enhanced protein stability, we analyzed clonogenicity of murine hematopoietic progenitors ectopically expressing (358-452) c-Myb. Even if these cells exhibited enhanced clonogenic potential *in vitro*, they were unable to induce a myeloproliferative syndrome in transplanted mice. Thus, we performed experiments to test the cooperation of this mutant with Bcl-2. We report here that co-expression of (358-452) c-Myb and Bcl-2 reduces the apoptosis susceptibility and enhances the proliferative potential of growth factor-deprived hematopoietic cells, compared to cells transduced with individual genes.

MATERIALS AND METHODS

Plasmids

MigRI-c-Myb-HA and MigRI- (358-452) c-Myb-HA were previously described [7].

The Bcl-2 cDNA was cloned in pLXSP as described in *Cirinnà et al., 2000* [18].

Cell lines and retroviral transductions

Mouse marrow cells were recovered from femurs and tibia of C57/BL6 mice and enriched for lineage-negative (Lin⁻) precursors using the StemSep Mouse Progenitor Enrichment Kit

(Stem Cell Technologies Inc., Vancouver, Canada). Lin⁻ Sca-1⁺ Kit⁺ cells were isolated by Fluorescence Activated Cell Sorting using PE-conjugated anti-Sca-1 and APC-conjugated anti-c-Kit antibodies (BD Biosciences, San Diego, CA, USA). Cells were kept in culture in StemSpan SFEM medium (Stem Cell Technologies Inc.) supplemented with IL-3 (6 ng/ml), Kit ligand (25 ng/ml), Flt3 ligand (50 ng/ml) and IL-6 (10 ng/ml). Cells were transduced with supernatant of either MigRI empty vector, MigRI-c-Myb-HA or MigRI- (358-452) c-Myb-HA. Retrovirally-transduced cells were selected by GFP-positive FACS sorting.

The murine IL-3 dependent 32Dcl3 myeloid precursor cell line was cultured and retrovirally transduced with MigRI empty vector, MigRI-c-Myb-HA or MigRI- (358-452) c-Myb as previously described [7]. Cells were then subsequently transduced with pLXSP-Bcl-2 and subjected to puromycin- resistance selection.

Clonogenic assays

For colony formation assays, 10⁴ retro-transduced Lin⁻ Sca-1⁺ Kit⁺ cells were plated in methylcellulose (Stem Cell Technologies Inc.) in the presence of optimal concentrations of cytokines as described above. After 7 days, colonies were counted. Replating assays were performed after methylcellulose solubilization in warm medium.

RESULTS

Effects of a stable c-Myb mutant on murine hematopoietic progenitors

In murine bone marrow, the Lin⁻ Sca-1⁺ Kit⁺ population is highly enriched for hematopoietic stem cells (HSC) [19]. These cells were purified and transduced with the MigRI empty vector, the full-length c-Myb or the (358-452) c-Myb to assess the biological consequences of expressing a c-Myb mutant with enhanced protein stability [7].

After GFP sorting, equal numbers of cells (10⁴) were plated in methylcellulose in the presence of IL-3, KL, Flt-3 ligand and IL-6 and colonies were scored 7 days later. Cells transduced with the MigRI empty vector or the full-length c-Myb retrovirus generated an approximately equal number of colonies; by contrast, cells transduced with (358-452) c-Myb were approximately 4-fold more clonogenic. Secondary and tertiary colony formation was also tested by replating cells obtained after methylcellulose solubilization. While number of colonies formed from cells transduced with MigRI empty vector decreased in secondary replating assays, cells transduced with full-length c-Myb showed an increased clonogenic potential. Moreover, cells transduced with (358-452) c-Myb formed 5-fold more colonies than cells transduced with wild-type c-Myb (Fig. 1A).

(358-452) (but not the full-length) c-Myb-expressing cells grew in liquid culture for more than 4 months and retained an undifferentiated (Sca-1⁺, c-Kit⁺) immunophenotype when assessed by flow cytometry (data not shown).

Since (358-452) c-Myb expressing Lin⁻ Sca-1⁺ cells grew in liquid culture in the presence of IL-3 only [7] and (358-452) c-Myb transduced Lin⁻ Sca-1⁺ Kit⁺ cells were markedly more clonogenic than full-length c-Myb expressing cells, we tested the leukemogenic potential of these cells *in vivo*. Up to 10⁶ MigRI empty vector or (358-452) c-Myb

transduced Lin⁻ Sca-1⁺ Kit⁺ cells were injected i.v. in 12 sublethally- irradiated syngenic mice. No signs of leukemia were detected in either group after 4 months of follow-up (data not shown).

Co-expression of c-Myb and Bcl-2 enhances the proliferation and reduces apoptosis susceptibility of 32D cells

Overexpression of Bcl-2 inhibits the pro-apoptotic effect of deregulated oncogenes, such as Myc, thus enhancing their oncogenic potential [17, 20]. Therefore we sought to assess if Bcl-2 overexpression could increase the colony forming potential of c-Myb expressing cells, by enhancing their survival.

32Dcl3 cells were transduced with either wild type or (358-452) c-Myb, alone or in combination with the Bcl-2 retrovirus. Proliferation assays were performed in liquid culture in the presence of different concentration (0–2 ng/ml) of rmIL-3 and cell numbers were assessed at different time points. Fig. 2 shows that at optimal IL-3 concentrations the number of cells co-expressing c-Myb and Bcl-2 increased if compared to that of cells transduced with c-Myb alone. In the absence of IL-3, the only cells able to grow for 48 hours were those transduced with (358-452) c-Myb.

To assess whether the increased proliferation of (358-452) c-Myb-expressing cells was caused by reduced apoptosis induced by IL-3 starvation, we measured hypodiploid DNA content by FACS analysis. Few apoptotic cells were detected in cultures of cells co-expressing Bcl-2 and c-Myb [wild type or (358-452) c-Myb] or in cultures of cells expressing (358-452) c-Myb. Cells expressing Bcl-2 or c-Myb alone were more protected than parental 32Dcl3 cells from apoptosis induced by growth factor deprivation but the effect was modest compared to co-expression of c-Myb and Bcl-2 or (358-452) c-Myb alone (Fig. 3).

DISCUSSION

Several studies have shown that the expression of c-Myb is elevated or deregulated in many cases of acute myeloid and lymphoid leukemia [21, 22, 23], but the oncogenic effects of c-Myb are, at best, modest and the underlying mechanisms unclear.

In this work, we investigated the biological effect of a more stable form of c-Myb in transduced murine hematopoietic progenitors. Compared to wild-type c-Myb, (358-452) c-Myb enhanced the clonogenic potential of Lin⁻ Sca-1⁺ Kit⁺ cells in primary and secondary methylcellulose assays. Cells expressing mutant c-Myb had the immunophenotype of hematopoietic progenitors and were essentially immortal since were maintained in culture in the presence of cytokines for several months; however, they were unable to induce leukemia in syngenic recipient mice. This may be due to *in vivo* susceptibility to apoptosis caused by non optimal cytokine availability or to a latent proapoptotic effect of c-Myb overexpression.

Previous studies demonstrated that overexpression of Bcl-2 inhibits the proapoptotic effect of deregulated oncogenes, such as Myc [15, 16]; thus, we analyzed the effect of the co-expression of Bcl2 and c-Myb.

Furthermore, Bcl-2 is a c-Myb target gene, and its expression is important for the maintenance of the pool of early progenitors, suggesting that there may be a cooperative effect from expressing these two genes.

Overexpression of wild type c-Myb in 32Dcl3 cells had a modest effect in protecting the cells from IL-3 deprivation-induced apoptosis, probably because apoptotic stimuli induced proteasome-dependent degradation of the protein. Consistent with this interpretation, ectopic expression of the degradation-resistant c-Myb mutant conferred resistance to apoptosis, similarly to the expression of exogenous Bcl-2 in wild-type or (358-452) c-Myb transduced cells.

The increased proliferation of (358-452) c-Myb-expressing cells probably reflects enhanced proliferation potential of cells escaping apoptosis induced by IL-3 deprivation.

Studies are now in progress to test the transforming potential of c-Myb and Bcl-2 *in vitro* and in leukemogenic assays in mice.

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Fig 1. Clonogenic assays of $\text{Lin}^- \text{Sca-1}^+ \text{c-Kit}^+$

$\text{Lin}^- \text{Sca-1}^+ \text{Kit}^+$ cells were purified from C57/BL6 mice, retro-transduced and MigRi empty vector, MigRi c-Myb-HA or MigRi (358-452) c-Myb-HA, GFP-sorted and plated (10^4 /plate) in methylcellulose in presence of IL-3, IL-6, Flt3 ligand and SCF. Colonies were counted after 7 days (panel A). Panel B shows secondary colony assays performed after solubilization of methylcellulose in warm medium. Colonies were counted after 14 days.

Fig 2. Proliferation assays of 32Dcl3

32Dcl3 cells were retro-transduced with MigRI empty vector, MigRI c-Myb-HA, MigRI (358-452) c-Myb-HA individually or in combination with pLXSP Bcl-2. At the end of the infection cells were washed from IL-3 and seeded in liquid culture (10^4 /ml) in the presence (panel A, 2ng/ml) or in absence (panel B) of IL-3.

Fig 3. DNA content analysis of parental and Bcl-2/c-Myb expressing 32Dcl3 cells
 Retro-transduced 32Dcl3 cells were washed from IL-3 and seeded in liquid culture ($10^4/\text{ml}$) in absence of IL-3. Propidium Iodide assay was performed after 24 hours.