

Dysregulated expression of Bcl-xL in CD34⁺ cells isolated from patients with refractory anemia with excess of blasts during megakaryocytic differentiation

We investigated whether autocrine transforming growth factor- β 1 (TGF- β 1) production modulates Bcl-xL expression during megakaryocytic differentiation of CD34⁺ cells isolated from normal individuals from patients with refractory anemia with excess of blasts (RAEB). We found that autocrine TGF- β 1 production up-regulates Bcl-xL expression in normal megakaryocytopoiesis, and that this pathway is altered in RAEB cells.

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In a previous study, we demonstrated that normal and myelodysplastic CD34⁺ cells ongoing to megakaryocytic commitment produce TGF- β 1, which exerts a facilitating effect on the megakaryocytic differentiation.¹ Interestingly, recent studies showed that in normal conditions Bcl-xL is upregulated in early megakaryocytes, while it is down-regulated in senescent megakaryocytes.² Moreover, the capacity of normal megakaryocytes to fragment into platelets is dependent on the regulated expression of Bcl-xL.³ The first aim of this study was to investigate whether the progressive increase in the Bcl-xL expression observed throughout the megakaryocytic differentiation is regulated by the autocrine production of TGF- β 1. Furthermore, considering that the impaired cell differentiation is the fundamental pathophysiologic abnormality of myelodysplastic syndromes (MDS), the second aim of this study was to explore whether a dysregulated expression of Bcl-xL could contribute to the defective megakaryocytic differentiation commonly observed in MDS.

Normal CD34⁺ cells were isolated from bone marrow samples from 4 normal marrow donors, as previously described.⁴ MDS CD34⁺ cells were isolated from 11 patients affected by refractory anemia with excess of blasts (RAEB). This series of patients was the same as that investigated for p15 methylation state in a previous study.¹ CD34⁺ cells were seeded at 30×10^3 /mL in megakaryocytic differentiating culture medium as previously described,¹ with and without the addition of neutralizing anti-human TGF- β 1 monoclonal antibody (35 mg/mL, R&D Systems, Minneapolis, MN). Cell medium (including the anti-TGF- β 1 monoclonal antibody) was replaced weekly. On days 7, 14 and 21 the CD41 expression was evaluated by flow cytometry (anti-CD41 fluorescein isothiocyanate-conjugated monoclonal antibody from Caltag Laboratories, Burlingame, CA, USA). A population of CD41⁺ cells more than 90% pure was isolated by immunomagnetic sorting, as previously described,^{1,4} and assayed for Bcl-xL expression using reverse transcriptase-polymerase chain reaction (RT-PCR). RNA extraction and cDNA synthesis were performed as previously described.⁴ After amplification with specific primers for Bcl-xL and β -actin,^{4,5} the mixture was separated on agarose gel and transferred onto nylon membranes (Amersham, Arlington Heights, IL, USA). Standard hybridization was performed using a Bcl-xL specific DNA probe, labeled with [α -³²P] dCTP (3,000 mCi/mmol; ICN). After autoradiography, densitometric analysis was conducted using the Molecular Imager (Biorad, Hercules, CA, USA). Bone marrow mononuclear cells, and water were used as positive and negative controls, respectively. Statistical analysis of data was performed by Mann Whitney's test and paired t-tests, as appropriate.

In normal CD34⁺ progenitor cultures we observed a progressive increase of the CD41⁺ cell output from day 0 to day 21 of culture (Figure 1A). Low levels of Bcl-xL mRNA were found in CD34⁺ cells. During megakaryocytic differentiation, in agreement with previous observations,² Bcl-xL isoform was increasingly

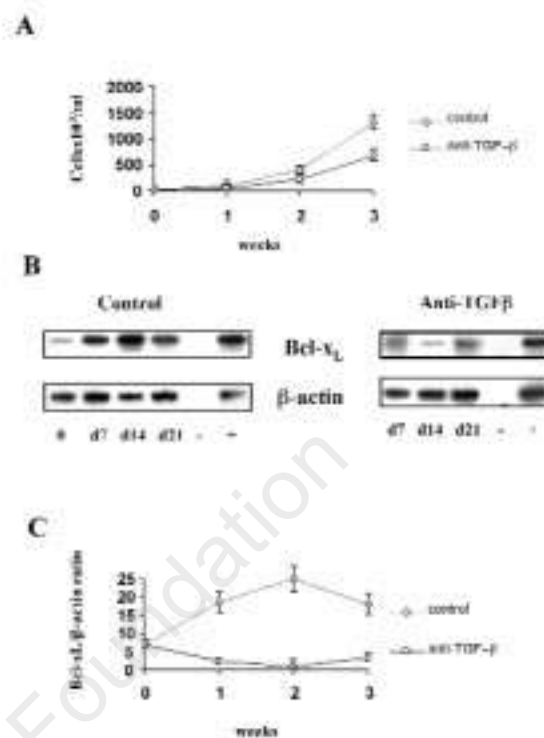


Figure 1. Bcl-xL expression and TGF- β 1 effect in normal megakaryocytic differentiation. (A) CD41⁺ cell generation from normal CD34⁺ cells seeded in megakaryocytic differentiating culture medium, with and without the addition of neutralizing anti-human TGF- β 1 monoclonal antibody. Anti-TGF- β 1 antibody induced a significant decrease of CD41⁺ cell number ($*p < 0.001$), without affecting total cell recovery. (B) Semiquantitative RT-PCR analysis of Bcl-xL mRNA in a representative experiment. Bcl-xL, expressed in CD34⁺ cells at baseline, progressively increased in CD41⁺ cells on day 7 and day 14, with a little decrement on day 21. Neutralizing anti-TGF- β 1 antibody induced a significant reduction of Bcl-xL mRNA on day 7 and day 14, with only a little increment on day 21. Negative and positive controls are indicated with - and +, respectively. (C) Bcl-xL/ β -actin ratios at different culture days in normal megakaryocytic differentiation (same experiment as in B).

expressed in CD41⁺ cells until day 14, with a slight decrement at day 21 (Figures 1B, 1C). When cells were grown in the presence of neutralizing anti-TGF- β 1 antibody, the CD41⁺ cell output at days 14 and 21 was significantly lower ($390.3 \pm 80.3 \times 10^3$ cells/mL and $220.8 \pm 47.6 \times 10^3$ cells/mL at day 14, respectively, $p=0.02$; $1303 \pm 52.8 \times 10^3$ cells/mL and $669 \pm 39.8 \times 10^3$ cells/mL at day 21, respectively, $p < 0.001$; mean cell count \pm SEM) (Figure 1A). Moreover, we observed a down-regulation of Bcl-xL expression until day 14, with a little increment at day 21 (Figure 1B, 1C). These results are consistent with the hypothesis that in normal hematopoiesis the autocrine production of TGF- β 1 by cells differentiating towards the megakaryocytic lineage facilitates the differentiation process by the Bcl-xL up-regulation. The CD41⁺ cell output from RAEB CD34⁺ cell cultures was significantly lower than in normal cell cultures at day 7 ($88.5 \pm 18.5 \times 10^3$ cells/mL and $28.2 \pm 6.1 \times 10^3$ cells/mL, mean cell count \pm SEM, respectively, $p < 0.05$), and only in two of the 11 samples could a low number

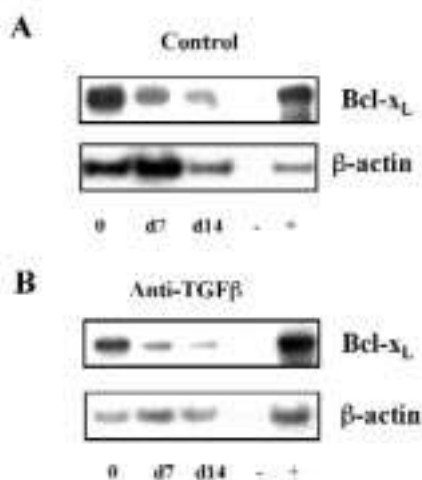


Figure 2. Semiquantitative RT-PCR analysis of Bcl-xL mRNA in one sample of RAEB cells (representative experiment). **A)** Bcl-xL was expressed at high levels in CD34⁺ cells on day 0, then dramatically decreased in CD41⁺ cells on day 7 and day 14. **B)** Neutralizing anti-TGF- β 1 antibody did not modify this altered pattern of Bcl-xL mRNA expression. Negative and positive controls are indicated with - and +, respectively.

of viable CD41⁺ cells be harvested at day 14 of culture. Bcl-xL mRNA was detected at high levels in all samples of RAEB CD34⁺ cells at day 0, and it appeared progressively down-regulated at days 7 and 14 of culture (Figure 2A).

Moreover, the Bcl-xL expression pattern was similar in control cultures and in cultures containing anti-TGF- β 1 neutralizing antibody (Figure 2B). The deregulated expression of Bcl-xL in MDS CD34⁺ cells was not dependent on impaired TGF- β 1 autocrine production. In fact, we have previously demonstrated that MDS CD34⁺ cells induced towards megakaryocytic differentiation produce TGF- β 1.¹

In conclusion, our data confirm that Bcl-xL up-regulation plays a fundamental role in normal megakaryocytopoiesis and demonstrate that it depends on the autocrine TGF- β 1 produced by differentiating cells. Furthermore, this study highlights that Bcl-xL expression in RAEB CD34⁺ cells undergoing megakaryocytic commitment is completely altered and suggests that it can play a role in their defective differentiation capacity.

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Aggressive natural killer cell leukemia: clinical features and treatment outcome

The clinical features of thirteen patients with very aggressive natural killer cell leukemia (ANKL) were retrospectively analyzed. The common symptoms and signs were fever (69.2%), B symptoms (100%), hepatosplenomegaly (76.9%), disseminated intravascular coagulopathy (76.9%), and hemophagocytosis (62%). Epstein-Barr virus (EBV) *in situ* hybridization was positive in 91%. Clinical response to treatment was observed in 38%, however, the median survival was 47days.

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Aggressive natural killer-cell leukemia (ANKL) is the rarest and least well characterized malignancy and the clinical features have been described only in a few case reports.¹⁻⁸ In this study, we conducted a retrospective study to investigate the natural history of cancer and the clinical outcome after treatment.

Between March 1994 and August 2001, 13 patients were diagnosed with ANKL. The diagnostic criteria were based on bone marrow infiltration of malignant cells with typical immunohistochemical features and characteristic clinical features. The cells were negative for sCD3 and positive for cCD3 and CD56. Tumor cells were negative for CD13 and CD33. Immunophenotypes of bone marrow samples were analyzed by flow cytometer using anti-CD2, -CD3, -CD4, -CD7, -CD8, -CD19, -CD16/56, and, anti-TdT antibodies. Immunostaining using CD30 and TIA-1 was performed on a core biopsy of bone marrow. Epstein-Barr virus (EBV) RNA was detected by an *in situ* hybridization (ISH) tech-