

Blocking the APRIL circuit enhances acute myeloid leukemia cell chemosensitivity

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ABSTRACT

Resistance to chemotherapy-induced cell death represents a major obstacle in the treatment of acute myeloid leukemia. APRIL (A Proliferation Inducing Ligand) is a member of the tumor necrosis factor superfamily that plays a key role in normal B-cell development, while promoting survival and proliferation of malignant B cells. We investigated APRIL expression and activity in acute myeloid leukemia. We found that APRIL mRNA and protein, including the secreted form, are expressed in leukemic cells of patients with M0, M2 and M4 acute myeloid leukemia subtypes but not in normal hematopoietic progenitors. Retrovirus-mediated APRIL expression in normal hematopoietic progenitors confers resistance to chemotherapeutic drugs-induced apoptosis. Conversely, blocking APRIL function by recombinant soluble APRIL receptors increased chemotherapeutic drugs-induced cell death in acute myeloid leukemia cells. These results indicate that APRIL acts in an autocrine fashion to protect acute myeloid leukemia cells from drug-induced death and foresee a therapeutic potential of APRIL antagonists in the treatment of acute myeloid leukemia.

Key words: acute myeloid leukemia, APRIL, chemosensitivity.

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Introduction

A key issue in the treatment of acute leukemia is the development of resistance to chemotherapy. The identification of the molecular mechanisms promoting the survival of leukemic cells is a mandatory step to improve the efficacy of therapeutic approaches. APRIL (a proliferation-inducing ligand), is a member of the TNF superfamily expressed in B-cell progenitors, monocytes, dendritic cells, and megakaryoblasts, which may contribute to the development of B-cell malignancies, including non-Hodgkin's lymphoma (NHL), B-CLL and multiple myeloma (MM), through the enhancement of cell survival and proliferation.¹⁻⁶ APRIL is a homotrimeric type 2 transmembrane protein that also exists in soluble form deriving from the intracellular cleavage of the full-length protein. It can bind with high affinity to two members of the TNF-receptor superfamily, the B-cell maturation antigen (BCMA) and the transmembrane activator and calcium modulator and cyclophilin ligand-interactor (TACI).^{1,7,8} However, APRIL binding was also shown on cells lacking known receptors, suggesting the existence of another specific interaction.^{1,8,9} In B-cell malignancies, the autocrine production of APRIL contributes to cancer cell survival and resistance to therapeutic drugs, as indicated by the increased sensitivity to dexamethasone and flavopiridol observed after neutralization of APRIL expression in B-lymphoma and B-CLL cells, respectively. The chemoresistance acquired by B-CLL cells through the expres-

sion of APRIL prompted us to study its involvement in the increased survival of acute myeloid leukemia (AML) cells. We show that AML blasts express considerable levels of APRIL protein and that its expression results in increased resistance to drug-induced apoptosis. APRIL neutralization, combined with chemotherapeutic drugs, leads to cell death induction in AML cells.

Design and Methods

Cell purification, culture and transduction

CD34⁺ hematopoietic progenitors were purified by peripheral blood as described.⁹ To induce unilineage granulocytic differentiation, CD34⁺ cells were cultured in serum-free medium supplemented with growth factors as previously described.¹⁰ Leukemia cells were obtained from the peripheral blood of adult patients with AML diagnosed at IOM (Catania, Italy) after obtaining their informed consent. Mononuclear cells (MNCs) were prepared by Ficoll-Paque density centrifugation. Cells were cultured in RPMI supplemented with 10% fetal bovine serum (GIBCO). Purity of blasts was assessed by staining with fluorescein isothiocyanate (FITC)-conjugated anti-CD34. All samples considered in this study were > 95% CD34⁺. APRIL cDNA was cloned into the pcDNA3.1 (by Invitrogen) or PINCO retroviral vector carrying the green fluorescent protein (GFP) as a reporter gene.⁹ Cell infection and sorting were performed as previously described.⁹

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The online version of this article contains a supplementary appendix.

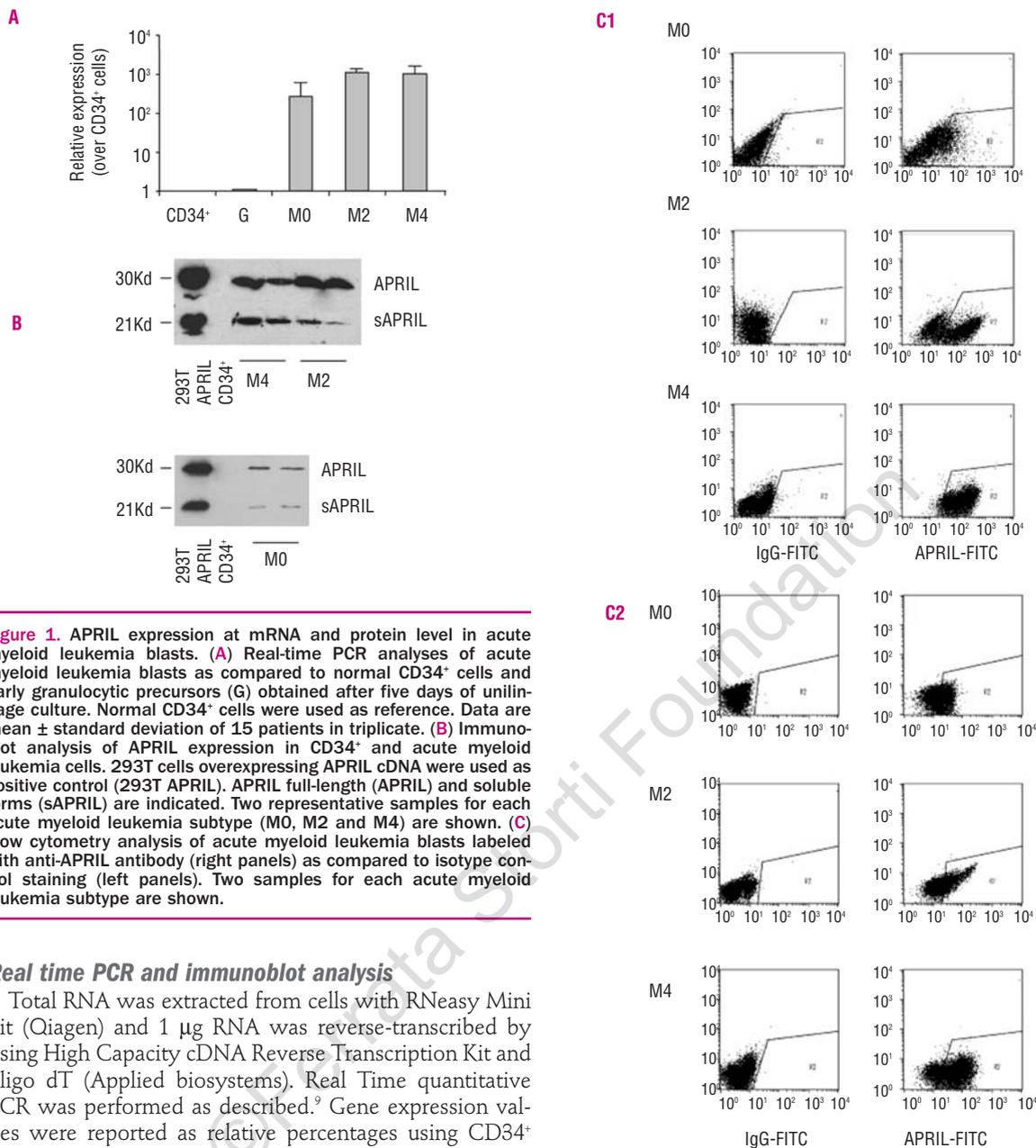


Figure 1. APRIL expression at mRNA and protein level in acute myeloid leukemia blasts. (A) Real-time PCR analyses of acute myeloid leukemia blasts as compared to normal CD34⁺ cells and early granulocytic precursors (G) obtained after five days of unilineage culture. Normal CD34⁺ cells were used as reference. Data are mean \pm standard deviation of 15 patients in triplicate. (B) Immunoblot analysis of APRIL expression in CD34⁺ and acute myeloid leukemia cells. 293T cells overexpressing APRIL cDNA were used as positive control (293T APRIL). APRIL full-length (APRIL) and soluble forms (sAPRIL) are indicated. Two representative samples for each acute myeloid leukemia subtype (M0, M2 and M4) are shown. (C) Flow cytometry analysis of acute myeloid leukemia blasts labeled with anti-APRIL antibody (right panels) as compared to isotype control staining (left panels). Two samples for each acute myeloid leukemia subtype are shown.

Real time PCR and immunoblot analysis

Total RNA was extracted from cells with RNeasy Mini kit (Qiagen) and 1 μ g RNA was reverse-transcribed by using High Capacity cDNA Reverse Transcription Kit and oligo dT (Applied biosystems). Real Time quantitative PCR was performed as described.⁹ Gene expression values were reported as relative percentages using CD34⁺ cells as reference control. Commercial ready-to-use primers/probe mixes were used (Assay-on-Demand Gene Expression products, Hs00601664_g1; Applied Biosystems). For immunoblotting, protein extracts were prepared by resuspending cell pellets in 1% nonidet P-40 lysis buffer and analyzed in equal amounts. Immunoblots were probed with rabbit anti-APRIL polyclonal antibody⁹ and with anti-actin monoclonal antibody (Sigma). For flow cytometry analysis of APRIL expression, cells were incubated for one hour on ice in PBS/1% BSA and with monoclonal anti-APRIL antibody (Aprily-1 Alexis), washed and incubated with a fluorescein isothiocyanate (FITC)-conjugated antimouse secondary antibody (Invitrogen-Molecular Probes, Eugene, OR, USA).

Drug treatment

Cells were seeded at a density of 1×10^5 cells/mL and incubated in the presence of chemotherapeutic agents. A series of dose-ranging studies were performed: 50-200

ng/mL for etoposide and camptothecin, 20-80 μ M for Ara C and 5-20 μ M for daunorubicine. Choice of the final drug doses was based on the sensitivity of the different subtypes of AML. The dosages were used in combination with 5 μ g/mL APRIL blocking receptors, BCMA-Fc and TACI-Fc or Fn14-Fc (Alexis). Cells were incubated overnight and viability was determined by both trypan blue exclusion and acridin orange/ethidium bromide staining, followed by fluorescence microscopy analysis.

Results and Discussion

We evaluated the expression of APRIL mRNA in 15 AML samples (>95% CD34⁺ blast cells), in normal hematopoietic CD34⁺ progenitors (HPCs), and in early granulocytic precursors generated from normal HPCs cultured in serum-free medium containing interleukin-3,

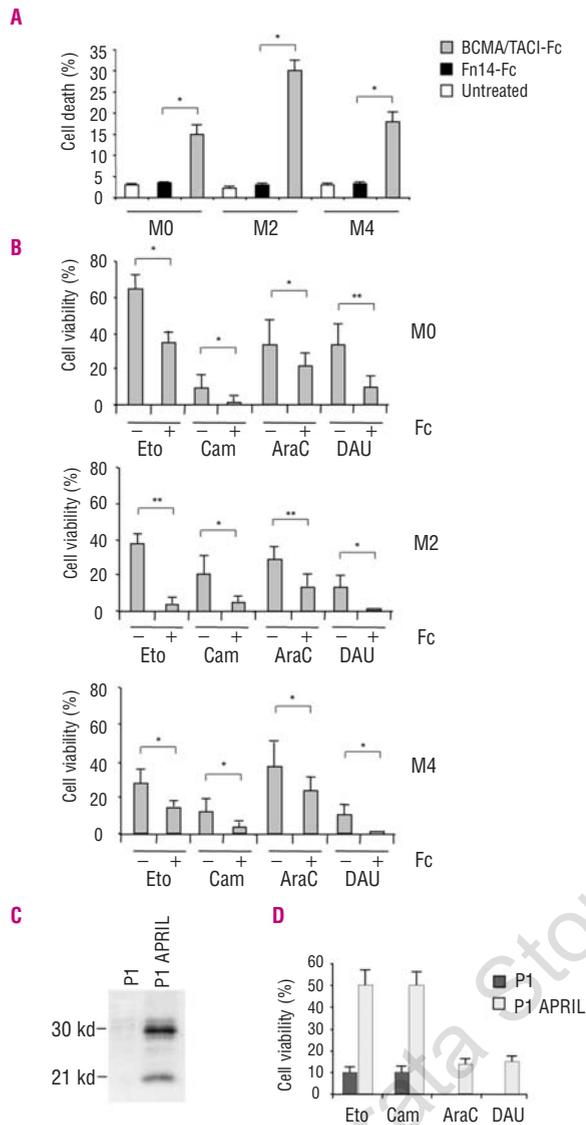


Figure 2. APRIL promotes the survival of acute myeloid leukemia cells. **(A)** acute myeloid leukemia blasts of different subtypes were treated with 5 μ g TACI-Fc and 5 μ g BCMA-Fc (BCMA/TACI-Fc) or 10 μ g Fn14-Fc recombinant soluble receptors. After 24 hours, cell viability was evaluated with acridin orange/ethidium bromide staining and fluorescence microscopy analysis. **(B)** Acute myeloid leukemia samples were treated for 24h with 50 ng/mL of etoposide (Eto), 200ng/mL of camptothecin (Cam), 40 μ M of cytosine arabinoside (AraC) or 10 μ M of daunorubicin (DAU). Data are mean \pm SD of 15 patients in triplicate, 5 for each subtype. * $0.01 < p < 0.05$; ** $p < 0.01$ two-tailed *t*-test. **(C)** Immunoblot analysis of CD34⁺ cells transduced with the PINCO empty vector (P1) or the PINCO vector containing APRIL cDNA (P1APRIL). **(D)** CD34⁺ cells transduced with P1 and P1APRIL were treated with camptothecin, etoposide, daunorubicin and AraC. Cell viability was evaluated with trypan blue counting after 12 hrs. of treatment. Data are mean \pm s.d of three independent experiments in triplicate.

GM-CSF and G-CSF.¹⁰ Real-time PCR analysis showed APRIL mRNA expression in all AML samples, including 5 cases of M0, 5 cases of M2 and 5 cases of M4 subtype by FAB classification, whereas normal CD34⁺ HPCs and early granulocytic precursors did not express APRIL (Figure 1A). APRIL expression was confirmed by Western blot analysis, which showed the presence of

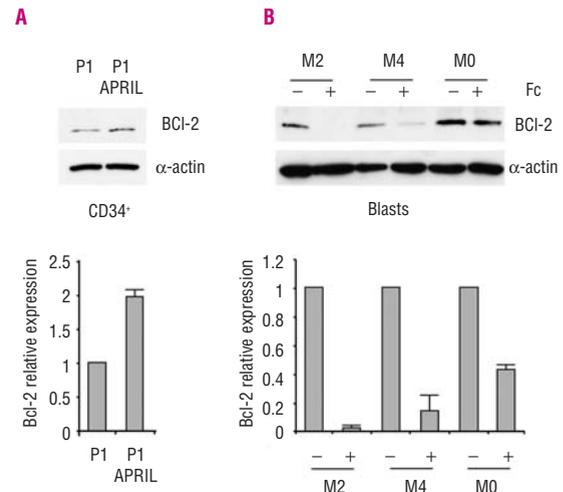


Figure 3. APRIL downstream signaling. **(A)** Western blotting of CD34⁺ cells transduced with P1 and P1APRIL. **(B)** Acute myeloid leukemia blasts of M2, M4 and M0 subtypes were treated with 5 μ g/L TACI-Fc and 5 μ g/L BCMA-Fc (BCMA/TACI-Fc) recombinant soluble receptors and lysated after 24 hrs. One representative of three independent experiments is shown.

both full length and soluble forms of the APRIL protein in AML blasts (Figure 1B). We next performed immunofluorescence experiments to determine the expression pattern of APRIL in myeloid leukemia blasts. In 21 cases analyzed, a significant percentage of the cells were stained by anti-APRIL antibodies (Figure 1C). The percentage of positive cells varied, being higher in the M4 and M2 subtypes than in M0 blasts (Figure 1C). In addition, we evaluated APRIL expression by FACS analysis in other FAB subtypes, including 2 M3 and 2 M5 (*Online Supplementary Figure S1A*). Overall, these data indicate that APRIL expression is specifically found in myeloid leukemia cells, with the exception of M3, but not in normal CD34⁺ cells or early granulocytic precursors. The anti-apoptotic activity showed by APRIL in B-CLL, suggests that the acquisition of APRIL expression in AML may generate an autocrine loop that could contribute to the leukemia transformed phenotype. To directly evaluate the pro-survival effect of APRIL expression on AML cells, we competitively inhibited APRIL activity with an excess of recombinant soluble receptors. Neutralization of the APRIL circuit resulted in significant induction of cell death in all the AML samples, particularly in the M2 subtype (Figure 2A), suggesting that APRIL acts as a survival factor in myeloid leukemia cells. To investigate whether APRIL neutralization results in AML cell apoptosis, we next evaluated annexin-V/7AAD staining on 3 different M2 samples treated for 24 hours with the blocking reagents. This treatment induced a considerable increase in annexin-V binding (*Online Supplementary Figures S1B and S1C*), indicating that constitutive expression of APRIL protects AML cells from apoptosis. Such an anti-apoptotic activity of APRIL in AML cells might contribute to the increase in resistance to chemotherapy. To investigate this hypothesis, we analyzed the cell death response of 15 fresh AML samples treated with etoposide or camptothecin, two widely used DNA-dam-

aging drugs. We treated the cells *in vitro* with either drug in the presence or absence of competing soluble APRIL receptors. The results indicate that inhibition of APRIL signaling significantly increased the apoptotic cell death induced by etoposide or camptothecin, independently of the subtype of the leukemia samples (Figure 2B). These data prompted us to study whether APRIL blocking agents have a synergistic effect with chemotherapeutic drugs currently used in AML. We, therefore, treated leukemia samples representative of the M0, M2 and M4 subtype with cytosine arabinoside (AraC) or daunorubicin, in the presence or absence of APRIL soluble receptors. As for others chemotherapeutic drugs, the block of APRIL significantly enhanced the induction of cell death by AraC or daunorubicin, independently of the AML subtype (Figure 2B). These data suggest that APRIL activity is part of AML blast response to cytotoxic agents and may play a relevant role in protecting the cells from cell death induced by exogenous stimulation, possibly contributing to chemotherapy resistance. To confirm that APRIL upregulation increases the resistance to chemotherapeutic drugs in myeloid progenitors, we transduced CD34⁺ HPCs with the retroviral PINCO vector carrying the APRIL gene together with a green fluorescent protein (GFP) reporter.⁹ After flow cytometry sorting, the virtually pure (>98%) transduced population was analyzed for APRIL expression, which was comparable with that observed in AML samples (Figure 2C and *data not shown*). In line with the results obtained in AML cells, exogenous APRIL expression in primary HPCs conferred a significant protection from etoposide, camptothecin, daunorubicin, and AraC treatment (Figure 2D). The anti-apoptotic protein Bcl-2 is induced by APRIL in B-cell lymphoma.^{2,3} Since high Bcl-2 expression is associated with poor chemotherapy response,^{11,12} we investigated the relationship between APRIL and Bcl-2 in AML. Western blotting analysis showed that exogenous expression of APRIL up-regulated Bcl-2 in CD34⁺ cells, whereas APRIL neutralization resulted in Bcl-2 downregulation in primary AML cells (Figure 3A, B), suggesting that APRIL protects AML from chemotherapeutic drugs through the upregulation of Bcl-2. Thus, APRIL expression promotes

the resistance to chemotherapy of normal myeloid progenitors, while APRIL neutralization considerably increases the cytotoxic activity of chemotherapeutic drugs against AML blasts. Transgenic expression of APRIL under the control of the Ick distal promoter induces the generation of lymphoid tumors in aged mice,¹³ indicating that the aberrant expression of APRIL in hematopoietic cells may promote the tumorigenic transformation. The antiapoptotic activity of APRIL has been previously demonstrated in B-lymphoma, multiple myeloma and B-CLL cells, but may be relevant in other cancers. In this study, we demonstrate that APRIL increases the resistance of AML cells to etoposide, camptothecin, daunorubicin, and AraC treatment, possibly by the upregulation of Bcl-2. Such an ability to promote the resistance to chemotherapeutic drugs in AML suggests a general oncogenic role for APRIL in hematologic malignancies. Biotechnology companies are devoting intense efforts to develop therapeutic molecules that target autoreactive or malignant B cells through the neutralization of APRIL or its partner Blys in autoimmune diseases, non-Hodgkin's lymphoma, multiple myeloma and B-CLL.^{8,14} In xenograft models of lung and colon cancer, intratumoral delivery of soluble BCMA was able to significantly reduce tumor growth.⁸ The increased cell death induced by chemotherapeutic drugs in AML cells upon inhibition of the APRIL circuit suggests that pharmacological strategies aimed at blocking of APRIL activity may considerably improve the efficacy of chemotherapeutic drugs in myeloid leukemia.

Authorship and Disclosures

All authors contributed significantly to the manuscript. MM, VC and AA conducted the *in vitro* experiments; MH provided anti-APRIL antibody for western blotting; CC collected human samples; DB planned experiments; MG provided intellectual input; DB, RDM, FG wrote the manuscript; RDM was responsible for research co-ordination and strategy. The authors reported no potential conflicts of interest.

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