Effect of cA2 Anti–Tumor Necrosis Factor-α Antibody Therapy on Hematopoiesis of Patients with Myelodysplastic Syndromes

Anna Boula,1 Michael Voulgarelis,2 Stavroula Giannouli,2 George Katrinakis,1 Maria Psyllaki,1 Charalambos Pontikoglou,1 Fotini Markidou,1 George D. Eliopoulos,1 and Helen A. Papadaki1

Abstract Purpose: Tumor necrosis factor α (TNF-α) plays a prominent role in the pathophysiology of myelodysplastic syndromes (MDS). The aim of this study was to explore the biological and immunoregulatory effect of the treatment with the anti–tumor necrosis factor-α monoclonal antibody cA2 on bone marrow (BM) progenitor/precursor and stromal cells and lymphocyte subsets, as well as the clinical response in MDS patients. Experimental Design: Ten low-intermediate risk MDS patients received i.v. cA2 (3 mg/kg) at weeks 0, 2, 6, and 12. The number, survival, and clonogenic potential of BM progenitor/precursor cells, the hematopoiesis-supporting capacity of BM stromal cells, and the lymphocyte activation status were investigated in the patients at baseline and following treatment using flow cytometry, clonogenic assays, and long-term BM cultures (LTBMC). Clinical response was evaluated according to standardized criteria. Results: cA2 administration reduced the proportion of apoptotic and Fas+ cells in the CD34+ cell compartment (P = 0.0215 and P = 0.0344, respectively) and increased the clonogenic potential of BM mononuclear and CD34+ cells (P = 0.0399 and P = 0.0304, respectively) compared with baseline. The antibody reduced tumor necrosis factor-α levels in LTBMC supernatants (P = 0.0043) and significantly improved the hematopoiesis-supporting capacity of LTBMC adherent cells. The proportion of activated peripheral blood and BM T-lymphocytes decreased significantly after treatment, suggesting an immunomodulatory effect of cA2. Two patients displayed minor hematologic responses whereas the remaining patients displayed stable disease with no disease progression. Conclusions: The encouraging biological insights from cA2 administration may be useful in conducting further clinical trials using cA2 for selected MDS patients, particularly those with evidence of immune-mediated inhibition of hematopoiesis.

Ineffective bone marrow (BM) hematopoiesis in myelodysplastic syndromes (MDS) is associated with increased apoptotic death of hematopoietic cells (1, 2). Excessive apoptosis has been mainly attributed to intrinsic defects within the clonal cell populations. Aberrant apoptotic cell death, however, has been shown to affect even the normal BM hematopoietic progenitors in MDS, indicating a possible apoptosis-inducing role of the marrow microenvironment (3). This might be due to altered adhesive interactions between the hematopoietic cells and the underlying stromal cells (4), presence of activated clone-directed T cells and macrophages that may affect both the normal and clonal BM subpopulations, relative deficiency of hematopoietic growth factors, or aberrant release of inhibitors (5).

Among the growth inhibitory cytokines, tumor necrosis factor α (TNF-α) has been reported to play a prominent role in the induction of intramedullary apoptosis directly and/or indirectly by up-regulating Fas antigen expression on hematopoietic progenitor cells (6). Increased levels of TNF-α have been identified in MDS patient sera and have been inversely correlated with the values of hemoglobin, response to erythropoietin treatment, disease severity, and overall survival (7–11). Aberrant TNF-α mRNA and protein expression has also been found in patients’ BM (1, 12) and local cytokine production has been reported to correlate to the levels of intramedullary apoptosis (13, 14), expression of Fas antigen on blast cells (12), and disease severity (15). The pathophysiologic significance of TNF-α in MDS has been further substantiated by the increase obtained in hematopoietic colony formation following in vitro blockade of the cytokine (12). Interestingly, in vivo neutralization of TNF-α activity using the soluble TNF receptor fusion protein etanercept or the anti-TNF-α chimeric monoclonal antibody (mAb) CA2 has resulted in substantial improvement of anemia of chronic disease (16–18) and occasional improvement of thrombocytopenia (19) in patients...
with other disease states (i.e., rheumatoid arthritis). Amelioration of anemia in these patients has been associated with down-modulation of the TNF-α-mediated apoptotic death of the erythroid progenitor and precursor cells in patients' BM (18).

The strong evidence implicating TNF-α in the pathophysiology of cytopenias in MDS and the experimental and clinical data showing a beneficial effect of TNF-α blockade in cytopenias associated with TNF-α-mediated disease states have encouraged the use of anti-TNF-α therapies in patients with MDS. A limited number of clinical trials using agents that directly inhibit the cytokine (i.e., etanercept or cA2) have been reported thus far (20–26) and results have shown a varying degree of efficacy. The main end point in these studies was the evaluation of clinical response, the safety, or the minimal effective dose of the selected agent. The effect of the treatment, however, on the survival characteristics of BM hematopoietic cells and the function of BM stromal cells has not been extensively studied.

In the current study, we have administered the mAb cA2 in a number of MDS patients and evaluated the number of the long-term culture initiating cells, the reserves and apoptotic characteristics of the BM erythroid and myeloid progenitor and precursor cells, and the hematopoiesis-supporting capacity of the long-term BM culture (LTBMC) stromal cells, before and after treatment in association to the clinical response. To probe further the mode of action of cA2 in MDS and because anti-TNF-α therapy has been reported to influence T-cell function, we have also looked for possible changes in the number and activation status of peripheral blood and BM lymphocyte subsets of the patients following treatment.

### Patients and Methods

#### Patients

Ten white patients with primary de novo MDS, five females and five males, ages 60 to 80 years (median, 71 years), were studied (Table 1). Diagnosis was based on morphologic abnormalities in the BM, cytopenia(s) in peripheral blood, and/or the presence of cytogenetic abnormalities. Informed consent according to the Helsinki protocol was obtained from all patients and the study had been approved by the Institutional Ethics Committee and the Hellenic Drug Organization. Patients with refractory anemia (RA), RA with ringed sideroblasts, and RA with excess of blasts (RAEB) according to the French-American-British classification (27) were eligible if they were RBC or platelet transfusion dependent and/or had hemoglobin levels <10.0 g/dL, platelet counts <100×10^9/L, and absolute neutrophil counts <1.5×10^9/L. Exclusion criteria included Karnofsky performance status <60%, liver or renal dysfunction defined by serum bilirubin or creatinine levels >34.2 and 176.8 μmol/L, respectively, cardiac failure with ejection fraction <45%, positive history for tuberculosis or positive tuberculin test, and recent infection.

Eligible patients were assigned to receive 3 mg/kg of cA2 (Remicade, infliximab; Schering-Plough, Athens, Greece) in a 2-hour i.v. infusion at weeks 0, 2, 6, and 12, without discontinuing previous medication with hematopoietic growth factors. Baseline and following treatment

### Table 1. Clinical and laboratory data of the patients studied

<table>
<thead>
<tr>
<th>UPN</th>
<th>Age (mo)</th>
<th>Duration (mo)</th>
<th>FAB</th>
<th>IPSS</th>
<th>BM blasts (%)</th>
<th>Peripheral blood counts</th>
<th>BM cellularity dependence</th>
<th>Transfusion dependence</th>
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<td>1</td>
<td>72/M</td>
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</table>

NOTE: Data were taken on the day of BM aspiration, before cA2 administration.

Abbreviations: UPN, unique patient number; FAB, French-American-British classification; IPSS, International Prognostic Scoring System; Hgb, hemoglobin; Neutro, neutrophils; Lympho, lymphocytes; Mono, monocytes; Plts, platelets; RARS, RA with ringed sideroblasts; RAEB, RA with excess of blasts.

1Units of RBCs required during the last 6 months.
2Values expressed as g/dL.
3Values expressed as ×10^9/dL.
and their subpopulations. Specifically, CD36

Theestimation of the CD34+/CD33+, CD34+/CD71+ and CD34+/CD61+ events using an Epics Elite model flow cytometer (Coulter, Miami, FL). Conjugated mouse immunoglobulin G of appropriate isotype served (QBEND-10, Beckman-Coulter, Marseille France) and anti-

with phycoerythrin-conjugated mouse anti-human CD34 mAb (StemCell Technologies, Inc., Vancouver, Canada) to obtain the bone marrow mononuclear cells (BMMMC).

**Purification of CD34+ cells**

CD34+ cells were isolated from BMMCs by indirect magnetic labeling (magnetic activated cell sorting; MACS isolation kit, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the protocol of the manufacturer. In each experiment, purity of CD34+ cells was >96% as estimated by flow cytometry.

**Reserves of the BM progenitor and precursor cells**

Flow cytometry was used to quantify the BM CD34+ progenitor cells and their subpopulations. Specifically, 1 × 106 BMMCs were stained with phycoerythrin-conjugated mouse anti-human CD34 mAb (QBEND-10, Beckman-Coulter, Marseille France) and anti-CD33 (D3H1.L60.251), anti-CD71 (YD1.2.2), or anti-CD61 (SZ21) FITC-conjugated mAbs (Beckman-Coulter). Phycoerythrin- or FITC-conjugated mouse immunoglobulin G of appropriate isotype served as negative controls. Data were acquired and processed on 500,000 events using an Epics Elite model flow cytometer (Couler, Miami, FL). The estimation of the CD34+/CD33−, CD34+/CD71− and CD34+/CD61− cells, representing the committed myeloid, erythroid, and megakaryocytic progenitor cells, respectively, was done in the gate of cells with low forward scatter and low side scatter properties (ref. 29; Fig. 1).

For the estimation of the erythroid precursor cells in the BM, 100 μL of BM cells were stained with anti-glycoporphine A (GlycoA)-phycoerythrin (11E4B7.6) and anti-CD36-phycoerythrin (FA6.152) mAbs (Beckman-Coulter) or with phycoerythrin-and FITC-conjugated mouse immunoglobulin G. RBC lysis and cell fixation were done using the Q-prep reagent system (Couler, Luton, United Kingdom). Data were acquired as above to identify the CD36+/GlycoA+ and CD36−/GlycoA+ early and mature erythroid precursor cells, respectively, in the gate of cells with low forward scatter and low side scatter properties (18, 30).

**Study of apoptosis**

Flow cytometry and 7-amino-actinomycin D staining (7AAD) were used to study apoptosis in the BM cell subpopulations. For the study of apoptosis in the CD34+ cell fraction, aliquots of 1 × 106 BMMCs stained with anti-CD34-phycoerythrin and anti-Fas-FITC (CD95; LOB Pharma AS, Oslo, Norway) to obtain the bone marrow mononuclear cells (BMMC).

**Clonogenic assays**

**Long-term culture initiating cells.** A limiting dilution assay was used for the quantification of the long-term culture initiating cells within the CD34+ cell fraction. In brief, CD34+ cells were overlaid on preformed murine MS-5 stromal layers at concentrations from 10 to 1,000 CD34+ cells per well in 96-well culture plates. Cultures were fed weekly by semi-depopulation, and after 5 weeks were overlaid with methylcellulose (StemCell Technologies, Inc., Vancouver, Canada) culture medium supplemented with 5 ng/mL granulocyte macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN), 50 ng/mL interleukin-3 (R&D Systems) and 2 IU/mL erythropoietin (Janssen-Cilag, Athens, Greece) as previously detailed (29). The frequency of long-term culture initiating cells was calculated by determining the CD34+ cell dilution that resulted in ≤37% wells negative for colonies (33).

**Erythroid and myeloid colony-forming units.** For the enumeration of the erythroid and myeloid colony-forming units, we cultured 105 BMMCs or 3 × 105 purified CD34+ cells, respectively, in 35-mm Petri dishes in 1 mL of methylcellulose culture medium supplemented, as previously detailed (29), in the presence of granulocyte macrophage colony-stimulating factor, interleukin-3, and erythropoietin as described above. Following 14 days of culture at 37°C-5% CO2 fully humidified atmosphere, colonies were scored and classified as erythroid burst-forming units (BFU-E) and total granulocyte- plus macrophage-plus granulocyte-macrophage colony-forming units (CFU-GM). The total sum was characterized as colony-forming cells (CFC).

**Megakaryocyte colony-forming units.** For the quantification of the megakaryocyte colony-forming units (CFU-Meg), we cultured 0.5 × 105 BMMCs or 2.5 × 105 purified CD34+ cells per chamber in a double-chamber slide using a supplemented commercially available culture medium (MegaCult-C, StemCell Technologies) according to the instructions of the manufacturer. Following 10 to 12 days of incubation at 37°C-5% CO2 humidified atmosphere, colonies were scored after fixation and staining of culture slides with anti-CD41 mAb (5B12, Dako, Glostrup, Denmark) using the alkaline phosphatase-antialkaline phosphatase technique as previously described (34). Results were expressed as total CFU-Meg colonies (pure CFU-Meg + mixed CFU-Meg).

**Assessment of BM stromal cell function**

**Standard LTBMCs.** BMMCs, 105, were grown according to the standard technique (35) in 10 mL of Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum (Gibco), 10% horse serum (Gibco), 100 IU/mL penicillin-streptomycin, 2 mmol L-glutamine, and 10−6 mol hydrocortisone sodium succinate (Sigma) and incubated at 33°C-5% CO2 humidified atmosphere. At weekly intervals, the cultures were fed by semi-depopulation and nonadherent cells were counted and assayed for CFCs as above.

**Cytokine measurement in LTBMC supernatants.** Cell-free supernatants of confluent LTBMCs (weeks 3-4) before and after cA2 treatment were stored at −70°C for TNF-α quantification using an ELISA kit. According to the manufacturer, the sensitivity of the assay is 0.09 pg/mL (Biosource International, Inc., Camarillo, CA).

**Recharged LTBMCs.** To test the hematopoiesis-supporting capacity of patient LTBMC stromal cells independently of the autologous cells, we used a two-stage culture procedure as previously described (36, 37). In brief, confluent LTBMC stromal layers from patients and normal controls were irradiated (10 Gy) and recharged with 5×104 murine MS-5 stromal layers at concentrations from 10 to 1,000 CD34+ cells per well in 96-well culture plates. Cultures were fed weekly by semi-depopulation, and after 5 weeks were overlaid with methylcellulose (StemCell Technologies, Inc., Vancouver, Canada) culture medium supplemented with 5 ng/mL granulocyte macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN), 50 ng/mL interleukin-3 (R&D Systems) and 2 IU/mL erythropoietin (Janssen-Cilag, Athens, Greece) as previously detailed (29). The frequency of long-term culture initiating cells was calculated by determining the CD34+ cell dilution that resulted in ≤37% wells negative for colonies (33).

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BM and peripheral blood lymphocyte subsets

Two-color flow cytometry was used for the analysis of BM and peripheral blood T-lymphocyte subpopulations. In brief, 100-μL aliquots of diluted BM or EDTA-anticoagulated peripheral blood samples were stained as described above with a combination of phycoerythrin- or FITC-conjugated mAbs (Beckman-Coulter). In particular, anti-CD3 (UCHT1), anti-CD8 (B9.11), or anti-CD4 (13B8.2) mAb was combined with each of the following mAbs representing T-cell activation markers: anti-HLA-DR (B8.12.2), anti-CD25 (interleukin-2 receptor; B1.49.9), anti-CD95 (Fas; UB2), anti-CD38 (T16), and anti-CD69 (TP1.55.3). Similarly, anti-CD19 (J4.119) mAb was combined with anti-CD23 (9P25) or anti-CD69 mAbs representing markers of B-cell activation. Analysis was done on 10,000 events in the gate of cells with low forward scatter and low side scatter properties. Results were expressed as proportions of cells expressing each mAb. Furthermore, by dividing the proportions of double-positive cells using the above-described mAb combinations by the percentages of total CD3+, CD4+, CD8+, or CD19+ cells, we estimated the proportions of activated cells within each lymphocyte subpopulation (38).

Statistical analysis

Data were analyzed in the GraphPad Prism statistical PC program (GraphPad Software, San Diego, CA). The Student’s t test for paired samples was used to define differences before and after treatment with cA2. Two-way ANOVA test was applied to define differences between patients and controls in the number of CFCs in recharged LTBMcs. Grouped data are expressed as mean ± SD.

Results

Reserves and survival characteristics of BM progenitor and precursor cells. The percentage of total CD34+ cells did not change significantly following treatment compared with baseline (P = 0.8195). Of the CD34+ cell subpopulations, a significant increase was obtained in the CD34+/CD33+ myeloid committed cells following treatment (0.56 ± 0.50) compared with baseline (0.32 ± 0.42; P = 0.0192). However, the proportion of CD34+/CD61+ megakaryocytic and CD34+/CD71+ erythroid progenitor cells remained unchanged after treatment compared with pretreatment values (P = 0.7768 and P = 0.3536, respectively), nor the proportion of total GlycoA+ erythroid precursor cells and their CD36+/GlycoA+ and CD36-/GlycoA- subpopulations displayed statistically significant difference after cA2 administration compared with baseline (P = 0.3536, P = 0.5032, and P = 0.7407, respectively).

The survival characteristics of patient BM progenitor cells before and after therapy are presented in Table 2. The proportion of apoptotic (7AADbright plus 7AADdim) cells in the ungated population (total BM cells) decreased significantly following cA2 therapy compared with pretreatment values (P = 0.0168). This decrease was mainly due to the lower proportion of apoptotic cells in the CD34+ cell fraction after therapy compared with baseline (P = 0.0215) because no statistically significant difference was found in the proportion of apoptotic cells in the total BM cell fraction excluding CD34+ cells, before and after therapy (P = 0.0582). The decrease in the percentage of apoptotic cells in the CD34+ cell fraction following therapy was associated with a significant reduction in the proportion of Fas+ cells (P = 0.0344). Overall, the reduction of apoptosis in the CD34+ fraction following treatment was due to the decrease obtained in the proportion...
of apoptotic cells within the CD34+/Fas + subpopulation compared with baseline \( (P = 0.0076) \) because no statistically significant difference was obtained in the proportion of apoptotic cells detected within the CD34+/Fas + subpopulation before and after therapy \( (P = 0.2352) \). Regarding the BM erythroid cells, a significant reduction was obtained in the proportion of apoptotic cells within the CD34+/CD71 + erythroid cell fraction following treatment \( (35.47 \pm 18.64\%) \) compared with baseline \( (54.06 \pm 26.25\% ; P = 0.0095) \) whereas no significant difference was obtained within the CD36+/GlycoA + \( (P = 0.0617) \) or the CD36+/GlycoA + \( (P = 0.0962) \) precursor cell populations. These findings are in agreement with the above-described data suggesting a decrease in the proportion of apoptotic cells in the CD34 + but not the non-CD34 + BM cell fraction following therapy.

**Clonogenic progenitor cells.** The number of long-term culture initiating cells that represent the best available approximation of primitive stem cells in the BM \( (39) \) did not change significantly in MDS patients following cA2 treatment \( (10.61 \pm 7.45 \text{ per } 10^4 \text{ CD34} + \text{ cells}) \) compared with baseline \( (10.11 \pm 6.51 \text{ per } 10^4 \text{ CD34} + \text{ cells}; P = 0.7893) \). A significant increase, however, was obtained in the number of CFCs obtained by BMMCs and CD34 + cells following treatment \( (2,770 \pm 1,547 \text{ per } 10^7 \text{ BMMCs and } 338 \pm 385 \text{ per } 5 \times 10^4 \text{ CD34} + \text{ cells}) \) compared with baseline \( (1,651 \pm 985 \text{ per } 10^7 \text{ BMMCs and } 186 \pm 213 \text{ per } 5 \times 10^4 \text{ CD34} + \text{ cells}; P = 0.0399 \) and \( P = 0.0304 \), respectively). This increase was due to the improvement of CFU-GM numbers \( (2,618 \pm 1,447 \text{ per } 10^7 \text{ BMMCs and } 292 \pm 290 \text{ per } 5 \times 10^4 \text{ CD34} + \text{ cells} \) posttreatment versus \( 1,447 \pm 900 \text{ per } 10^7 \text{ BMMCs and } 146 \pm 109 \text{ per } 5 \times 10^4 \text{ CD34} + \text{ cells} \) pretreatment; \( P = 0.0298 \) and \( P = 0.0441 \), respectively) because no significant difference was obtained in BFU-E numbers \( (160 \pm 70 \text{ per } 10^7 \text{ BMMCs and } 47 \pm 100 \text{ per } 5 \times 10^4 \text{ CD34} + \text{ cells} \) after therapy versus \( 204 \pm 222 \text{ per } 10^7 \text{ BMMCs and } 41 \pm 113 \text{ per } 5 \times 10^4 \text{ CD34} + \text{ cells} \) after therapy; \( P = 0.514 \) and \( P = 0.270 \), respectively). A significant increase was also obtained in the frequency of CFU-Meg after therapy \( (129 \pm 94 \text{ per } 5 \times 10^7 \text{ BMMCs and } 162 \pm 80 \text{ per } 2.5 \times 10^4 \text{ CD34} + \text{ cells}) \) compared with baseline \( (63 \pm 36 \text{ per } 5 \times 10^7 \text{ BMMCs and } 74 \pm 32 \text{ per } 2.5 \times 10^4 \text{ CD34} + \text{ cells}; P = 0.016 \) and \( P = 0.002 \), respectively). Cumulative and individual colony data before and after therapy are presented in Fig. 2A.

**BM stromal cell function.** Typical confluent stromal layers, mimicking the BM microenvironment and consisting of cells of hematopoietic and mesenchymal origin, were formed over the first 3 to 4 weeks in patient LTBMCs before and after therapy. The number of CFCs in the nonadherent cell fraction increased significantly following cA2 administration compared with baseline \( (P < 0.05) \) over a period of 5 weeks of culture (Fig. 2B). This increase may reflect the above-described improvement in the number and clonogenic potential of the myeloid progenitor cells after therapy but may also indicate an improvement in the hematopoiesis-supporting capacity of LTBMC adherent cells after therapy. To investigate this hypothesis, we evaluated the capacity of irradiated LTBMC stromal layers from the patients to support the growth of normal CD34 + cells before and after therapy. Before cA2 administration, the CFC recovery from nonadherent cells was significantly lower in the patients compared with controls \( (F = 5.144 > F_{0.05}; P < 0.05) \), suggesting defective hematopoiesis-supporting capacity of patient stromal cells. Following therapy, however, the number of CFCs in the nonadherent cell fraction did not differ significantly between patients and healthy controls \( (F = 0.534 < F_{0.05}; P > 0.05) \), suggesting a substantial improvement in the capacity of patient stromal layers to support hematopoiesis. In keeping with the CFC data was the significant decrease in the levels of TNF-α in LTBMC supernatants of patients after therapy \( (2.46 \pm 2.23 \text{ pg/mL}) \) compared with baseline \( (7.96 \pm 5.60 \text{ pg/mL}; P = 0.0043) \). Individual patient TNF-α levels before and after therapy are presented in Fig. 2C.

### Table 2. Study of apoptosis of BM cells before and after cA2 administration

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<th>Before cA2 treatment ((n = 10))</th>
<th>After cA2 treatment ((n = 10))</th>
<th>(P^*)</th>
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<td>Total BM cells</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>% 7AAD + cells</td>
<td>16.27 ± 11.81</td>
<td>8.08 ± 5.12</td>
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</tr>
<tr>
<td>Median (range)</td>
<td>14.35 (4.20-42.20)</td>
<td>7.20 (4.10-22.20)</td>
<td></td>
</tr>
<tr>
<td>BM cells minus CD34 + cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% 7AAD + cells</td>
<td>13.44 ± 11.57</td>
<td>6.93 ± 6.09</td>
<td>0.0582</td>
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<tr>
<td>Median (range)</td>
<td>9.90 (3.10-38.00)</td>
<td>3.70 (3.10-22.20)</td>
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<tr>
<td>CD34 + cell fraction</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>% 7AAD + cells</td>
<td>35.60 ± 20.97</td>
<td>20.54 ± 12.69</td>
<td>0.0215</td>
</tr>
<tr>
<td>Median (range)</td>
<td>31.70 (8.50-75.70)</td>
<td>20.60 (5.50-36.40)</td>
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<tr>
<td>Fas + cells</td>
<td>34.95 ± 16.13</td>
<td>18.43 ± 10.62</td>
<td>0.0344</td>
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<tr>
<td>Median (range)</td>
<td>43.10 (7.10-56.10)</td>
<td>16.60 (4.50-42.60)</td>
<td></td>
</tr>
<tr>
<td>CD34 + /Fas + cell fraction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% 7AAD + cells</td>
<td>43.88 ± 12.65</td>
<td>23.36 ± 24.50</td>
<td>0.0076</td>
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<tr>
<td>Median (range)</td>
<td>41.35 (27.00-64.20)</td>
<td>10.55 (5.40-66.70)</td>
<td></td>
</tr>
<tr>
<td>CD34 + /Fas + cell fraction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% 7AAD + cells</td>
<td>19.14 ± 15.25</td>
<td>12.90 ± 9.01</td>
<td>0.2352</td>
</tr>
<tr>
<td>Median (range)</td>
<td>13.40 (3.60-44.70)</td>
<td>13.60 (11.0-30.10)</td>
<td></td>
</tr>
</tbody>
</table>

Note: Values are expressed as means ± SD.

\(^*\) Statistical analysis was done using the Student’s \(t\) test for paired samples. \(P \leq 0.05\) was considered as statistically significant.
Peripheral blood and BM lymphocyte subsets. It has been reported that treatment with cA2 may display immunoregulatory effects in peripheral blood lymphocyte subsets of patients with rheumatic diseases (40–42). Because activated T-lymphocytes have been implicated in the pathophysiology of MDS (43), we studied the expression of activation markers in BM and peripheral blood T-lymphocyte subsets before and after treatment with cA2. Results are shown in Table 3. The proportion of BM CD3+ cells did not change significantly following therapy (70.68 ± 9.43%) compared with baseline (66.89 ± 7.02%; \( P = 0.323 \)), whereas the proportion of CD4+ and CD8+ cells displayed significant differences following therapy (\( P = 0.228 \) and \( P = 0.715 \)). However, the proportions of activated T-cells decreased significantly after treatment as was indicated by the

Fig. 2. Clonogenic progenitor cells and LTBM data before and after cA2 administration. A, left, columns, mean CFU-Meg, CFU-GM, and BFU-E numbers obtained with 10^7 BMNCs using clonogenic assays before and after treatment with cA2; bars, SE. Right, columns, mean colony values obtained with 5 × 10^4 CD34+ cells from the patients before and after therapy; bars, SE. Individual results of CFU-Meg and CFU-GM numbers before and after therapy are shown over the respective columns. Comparisons between values before and after therapy were done using the Student’s \( t \) test for paired samples. B, points, mean frequency of CFCs detected weekly in the nonadherent cell fraction of patient LTBM data before and after cA2 administration; bars, SE. Comparison between colony numbers before and after treatment time course was done by means of the two-way ANOVA. C, points, individual TNF-\( \alpha \) levels in patient LTBM supernatants harvested on confluence (weeks 3–4) before and after therapy with cA2. Comparison was done using the Student’s \( t \) test for paired samples.
percentage of Fas+ (P = 0.0002), HLA-DR+ (P = 0.0020), CD25+ (P < 0.0001), CD38+ (P = 0.0004), and CD69+ (P = 0.0002) cells detected in the CD3+ cell fraction. The decrease in the expression of activation markers was in both the CD4+ and CD8+ subpopulations (Fig. 3). In keeping with the BM data compared with baseline (70.17 ± 7.21; P = 0.970). However, the proportion of activated peripheral blood CD3+ cells decreased significantly after therapy as was indicated by the expression of Fas (P = 0.0015), HLA-DR (P = 0.0014), CD25 (P = 0.0022), CD38 (P < 0.0001), and CD69 (P = 0.0021) markers and this decrease was in both the CD4+ and CD8+ cells.

**Response evaluation.** Cytogenetic response and hematologic improvement were assessed according to standardized criteria (28). No cytogenetic response was identified using conventional cytogenetic techniques in any of the patients studied. Patient #7 displayed a minor erythroid response (hematologic improvement-erythroid; defined by ≥50% increase in platelet counts with a net increase between 10,000/μL and 30,000/μL) because his platelet counts elevated to 19,000/μL in serial measurements. Responses have lasted at least 2 months after the last dose of cA2. The remaining patients displayed stable disease on red cell, platelet, or absolute neutrophil counts and transfusion requirements. None of the patients displayed increase in blast counts, progression to RA with excess of blasts in transformation, or evolution to acute myeloid leukemia.

**Discussion**

There has been increasing interest during the last years for identification of novel therapeutic strategies for MDS targeting not only the malignant clone but also the BM microenvironment. These therapies are composed of inhibitors of DNA methyltransferase, farnesyl transferase and histone deacetylase inhibitors, arsenic trioxide, differentiating agents, immuno-modulatory and immunosuppressive drugs, and antiangiogenic and antiapoptotic agents alone or in combination (44–47).

Because TNF-α has been recognized to play a significant role in the pathophysiology of MDS, there is strong rationale for the use of therapeutic agents that may directly neutralize the effects of TNF-α, such as the soluble TNF-α receptor fusion protein etanercept and the chimeric anti-TNF-α mAb cA2. Results from a relatively limited number of clinical trials investigating the safety and efficacy of etanercept in the treatment of MDS have shown modest responses (20, 21, 23, 24, 27) that might increase following combination with antithymocyte globulin (26). Responses seem to be more promising with the neutralizing anti-TNF-α antibody cA2. Specifically, a preliminary report has indicated sustained erythroid responses in two patients with low-intermediate 1 risk MDS following six doses of cA2 (22) whereas a definite report comprising 28 patients with low-intermediate 1 risk MDS has shown sustained

### Table 3. Flow cytometric analysis of BM and peripheral blood lymphocyte subsets

<table>
<thead>
<tr>
<th></th>
<th>Before cA2 treatment</th>
<th>After cA2 treatment</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BM CD3+ cell fraction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Fas+ cells</td>
<td>37.62 ± 11.07</td>
<td>19.95 ± 12.36</td>
<td>0.0002</td>
</tr>
<tr>
<td>Median (range)</td>
<td>39.37 (25.44-57.70)</td>
<td>18.48 (3.80-47.40)</td>
<td></td>
</tr>
<tr>
<td>% HLA-DR+ cells</td>
<td>17.64 ± 7.42</td>
<td>11.57 ± 9.68</td>
<td>0.0020</td>
</tr>
<tr>
<td>Median (range)</td>
<td>16.41 (6.32-29.53)</td>
<td>8.66 (2.10-27.09)</td>
<td></td>
</tr>
<tr>
<td>% CD25+ cells</td>
<td>10.64 ± 1.84</td>
<td>5.37 ± 3.70</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Median (range)</td>
<td>9.84 (9.13-15.00)</td>
<td>4.30 (2.33-12.70)</td>
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</tr>
<tr>
<td>% CD38+ cells</td>
<td>30.47 ± 6.78</td>
<td>20.12 ± 4.41</td>
<td>0.0004</td>
</tr>
<tr>
<td>Median (range)</td>
<td>33.80 (18.00-35.16)</td>
<td>19.73 (11.70-26.18)</td>
<td></td>
</tr>
<tr>
<td>% CD69+ cells</td>
<td>48.27 ± 12.30</td>
<td>35.11 ± 15.05</td>
<td>0.0002</td>
</tr>
<tr>
<td>Median (range)</td>
<td>54.60 (29.87-66.02)</td>
<td>34.38 (14.60-61.80)</td>
<td></td>
</tr>
<tr>
<td><strong>Peripheral blood CD3+ cell fraction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Fas+ cells</td>
<td>47.12 ± 13.76</td>
<td>27.75 ± 7.35</td>
<td>0.0015</td>
</tr>
<tr>
<td>Median (range)</td>
<td>44.21 (26.1-65.4)</td>
<td>29.24 (14.69-38.50)</td>
<td></td>
</tr>
<tr>
<td>% HLA-DR+ cells</td>
<td>12.38 ± 3.80</td>
<td>6.27 ± 4.21</td>
<td>0.0014</td>
</tr>
<tr>
<td>Median (range)</td>
<td>12.30 (2.97-16.27)</td>
<td>7.72 (1.25-13.03)</td>
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</tr>
<tr>
<td>% CD25+ cells</td>
<td>9.58 ± 1.96</td>
<td>6.44 ± 2.65</td>
<td>0.0022</td>
</tr>
<tr>
<td>Median (range)</td>
<td>9.17 (7.64-13.70)</td>
<td>5.80 (1.97-11.87)</td>
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</tr>
<tr>
<td>% CD38+ cells</td>
<td>32.14 ± 9.30</td>
<td>20.40 ± 9.86</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Median (range)</td>
<td>30.10 (18.42-52.33)</td>
<td>15.45 (11.71-41.43)</td>
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</tr>
<tr>
<td>% CD69+ cells</td>
<td>36.45 ± 14.30</td>
<td>18.17 ± 6.13</td>
<td>0.0022</td>
</tr>
<tr>
<td>Median (range)</td>
<td>32.90 (19.21-53.80)</td>
<td>17.80 (8.78-28.12)</td>
<td></td>
</tr>
</tbody>
</table>

*Comparison was done with the Student’s t test for paired samples.

**NOTE:** All values are expressed as mean ± SD.
multilineage improvements and even cytogenetic responses in a significant proportion of patients after four cycles of treatment (25). The biological effect of cA2 on the functional characteristics of patient BM cells, however, has not been thoroughly investigated. In the current study, we have investigated the mode of action of cA2 in the BM of MDS patients by exploring the effect of the treatment on both BM hematopoietic and microenvironment cells. We have also explored for possible immunomodulatory effects of the treatment looking for alterations in the activation status of BM and peripheral blood lymphocytes following therapy.

We have found that treatment with four doses of cA2 at 3 mg/kg does not influence the frequency of long-term culture initiating cells, the proportion of total CD34+ cells, or the percentage of erythroid progenitor and precursor cells in patients’ BM. However, cA2 treatment increased significantly the clonogenic potential of the myeloid and megakaryocytic progenitor cells in the BMMC and CD34+ cell fraction. The beneficial effect of cA2 on the CFU-GM and CFU-Meg colony formation in our patients was associated with a significant improvement in the survival characteristics of cells within the CD34+ cell compartment. Specifically, following treatment, a significant reduction was observed in the proportion of apoptotic cells detected in the CD34+ cell fraction that was paralleled with significant down-regulation of Fas in this particular cell compartment. Interestingly, reduction of apoptosis was observed in the CD34+/Fas+ but not the CD34+/Fas- cell fraction, highlighting, therefore, the prominent role of Fas in the apoptotic depletion of hematopoietic progenitor cells in MDS (12). Notably, no statistically significant difference was documented in the proportion of apoptotic cells detected in the non-CD34+ BM cell fraction following treatment compared with baseline, indicating that the antiapoptotic effect of cA2 in MDS concerns specifically the CD34+ cells. In keeping with this finding was the effect cA2 on the survival characteristics of BM erythroid cells. As was anticipated on the basis of the above-described antiapoptotic effect of cA2 on CD34+ cells, a significant reduction was observed in the proportion of apoptotic cells detected in the CD34+/CD71+ cell fraction comprising the early erythroid progenitor cells. However, no significant difference was documented following treatment in the proportion of apoptotic cells detected in the GlycoA+ cell compartment representing the more mature stages of the erythroid development. Consistent with the unchanged survival

![Fig. 3. BM and peripheral blood activated lymphocytes in MDS patients before and after treatment with cA2. Columns, mean proportion of cells expressing activation markers within the BM (top) and peripheral blood (bottom) CD4+ and CD8+ cell fractions of the MDS patients studied before and after therapy with cA2; bars, SE. Comparisons were done using the Student’s t test for paired samples.](image-url)
characteristics of GlycoA+ cells following treatment was the erythroid colony formation by BMMCs or CD34+ cells that did not display any significant difference compared with baseline. Our data on the antiapoptotic effect of cA2 on CD34+ cells are in accordance with the findings of Stasi and Amandori (22), who also observed a remarkable decrease in the percentage of apoptotic (Annexin V–positive) BM CD34+ cells in two MDS patients treated with cA2; however, the number of parameters was too low to exert statistical results.

Regarding the effect of cA2 on BM microenvironment cells, we found a significant reduction in the levels of TNF-α in LTBMCS supernatants following treatment, suggesting a remarkable reduction of cytokine production by BM microenvironment cells. This decrease in supernatant TNF-α concentrations was associated with an amelioration of the hematopoiesis-supporting function of LTBMCS adherent layers following treatment on both the autologous and normal hematopoietic progenitor cell growth. Notably, down-modulation of TNF-α production by LTBMCS adherent layers following cA2 administration has also been reported in patients with active rheumatoid arthritis and has been associated with a significant improvement in the function of patient BM stromal cells (32).

It has been reported that cA2 may neutralize not only the soluble but also the transmembrane form of TNF-α on the cytokine-producing cells and can therefore activate the anti-body-dependent and complement-dependent cytotoxic mechanisms resulting in lysis of the TNF-α producing/expressing cells, mainly the activated macrophages (48). This mode of action of cA2 may give an explanation for the reduction of TNF-α production by patient LTBMCS adherent layers consisting of monocytes/macrophages at a significant proportion (35) and may also explain the significant reduction of activated BM and peripheral blood T-lymphocyte subsets following therapy. In particular, the possible cA2-mediated elimination of activated monocytes/macrophages in the patients may lead to down-regulation of antigen presentation process and, therefore, may result in down-regulation of immune responses generating activated T-cells. Notably, a more general immunoregulatory action of cA2 not attributable to TNF-α suppression alone but also to an indirect, but still elusive, effect on T-lymphocyte function has been reported in patients with rheumatoid arthritis treated with the antibody (49). An effect, however, on T-cell activation status of patients receiving cA2 has not been reported thus far.

The main goal of this study was to investigate the effect and mode of action of cA2 on BM progenitor/precursor and stromal cells and lymphocyte subsets in a limited number of MDS patients. Despite the statistically significant beneficial effect of cA2 on the survival characteristics and function of BM progenitor and microenvironment cells and the status of activation of BM and peripheral blood lymphocytes in these patients, the sample size is relatively small to exert conclusions about clinical response to the treatment.

In summary, we have investigated the biological and immunomodulatory effects of the treatment with the anti-TNF-α mAb cA2 in patients with MDS. We have shown that treatment with cA2 down-regulates the Fas-mediated apoptotic depletion of BM CD34+ cells, increases the clonogenic potential of the myeloid and megakaryocytic progenitors, and suppresses the production of TNF-α by BM microenvironment cells, ameliorating therefore the hematopoiesis-supporting capacity of BM stroma. Treatment with cA2 seems to significantly decrease the proportion of activated T- lymphocytes in both BM and peripheral blood. Although treatment under the current schedule retained a stable disease state rather than inducing a beneficial effect, the encouraging biological insights from this study may seem to be useful in conducting clinical trials using cA2 alone or in combination with other agents for preselected MDS patients, particularly those with clinical and/or laboratory data suggestive of immune-mediated inhibition of hematopoiesis.

Acknowledgments

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References


Effect of cA2 Anti–Tumor Necrosis Factor-α Antibody Therapy on Hematopoiesis of Patients with Myelodysplastic Syndromes

Anna Boula, Michael Voulgarelis, Stavroula Giannouli, et al.


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