Letter to the Editor

Pharmacological doses of all-*trans* retinoic acid promote G0/G1 transition and G1 arrest of normal human marrow CD34⁺ cells

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Dear Editor,

In myelodysplasia (MDS), a clone from defective pluripotent stem cells tends to dominate the marrow.¹ Progenitors from the abnormal clone exhibit ineffective haematopoiesis *in vitro* and *in vivo*,² and fail to generate fully functional neutrophils or sufficient mature red cells. This has prompted an intensive search for ways to circumvent the differentiation block in this condition. All *trans*-retinoic acid (ATRA) has attracted much interest as a potential enhancer of neutrophilic differentiation in MDS, since the demonstration that it is an effective inducer of differentiation in HL60 cells.³ Retinoid therapy (13-*cis* and all-*trans*) has been attempted in MDS, but the effectiveness was limited when used alone.^{4–6} This could be explained in part by the effects of pharmacological doses of retinoids on progenitors from normal resting haematopoiesis.

ATRA is a powerful inducer of terminal differentiation and cell cycle arrest of several myeloid cell lines *in vitro*. The process of terminal differentiation is closely associated with growth arrest in the G0/G1 phase of the cell cycle, and little is known of the effects of ATRA on the cell cycle of normal marrow progenitors. The purpose of our study was to evaluate concomitantly the cell cycle status (G0, G1 and S/G2/M phases) and the differentiation of normal marrow CD34⁺ cells exposed *in vitro* to pharmacological doses of ATRA.

Normal bone marrow cells were obtained after written consent from sternum puncture of eight patients undergoing cardiac surgery. Mononuclear cells were separated by centrifugation over Ficoll–Hypaque and enriched in CD34⁺ cells by direct immunoseparation using magnetic beads (DynaL CD34 Progenitor Cell Selection System[®], Dynal AS, Oslo, Norway). Purity of the selected cells was verified with HPCA-2 monoclonal antibody (mAb) (anti-CD34 R-PE directly conjugated; Becton-Dickinson, Mountain View, CA, USA), and was >90% in all experiments.

CD34⁺ cells were incubated for 7 d at 37°C at a concentration of 2×10^4 cells/ml in 5% CO₂ in a serum-free medium consisting of Iscove's medium supplemented with 1% deionized bovine serum albumin, 0.3 mg/ml human transferrin, 7.8 µg/µl cholesterol, 30 µg/µl L-α-phosphatidyl cholin, 0.25 U/ml human insulin, 0.1 mM 2-mercaptoethanol, 50 ng/ml SCF, 10 ng/ml IL-3 and 10 ng/ml IL-6. ATRA (1 µM) was added to this medium at d0 or d3, and counting of viable cells was performed by Trypan blue exclusion.

Cell cycle status (G0, G1 and S + G2/M) was determined at d0, d3 and d7 by simultaneous two-parameter analysis using

DNA content *versus* antibody staining for the Ki67 antigen.⁷ First, cell surface staining was performed using PE-conjugated HPCA-2 for 30 min. Isotype control was undertaken in parallel for each sample. After surface staining was complete, cells were fixed with 1% paraformaldehyde (30 min, 4°C) and permeabilized with 0.2% Triton X-100 (overnight, 4°C). Cells were then stained with FITC-conjugated MIB-1 (anti Ki67) and 0.5 µg/ml 7-aminoactinomycin D (7-AAD) overnight before analysis. Samples were run in low differential pressure in order to minimize CV, and at least 10⁴ cells were collected. FL1 (FITC) and FL2 (PE) fluorescence was collected on a log scale, and FL3 (7-AAD) fluorescence on a linear scale. Samples were analysed first by gating on forward scatter (FSC) *versus* side scatter (SSC) to select live cells. SSC *versus* CD34 fluorescence was then visualized and gates were established to define the subpopulations of interest. Finally, after gating on FL3 area *versus* FL3 width to eliminate aggregates, 7-AAD *versus* Ki67 was visualized, and the proportion of events corresponding to each cycle stage was determined.

The effects of ATRA on the differentiation of CD34⁺ cells were evaluated concomitantly using clonogenic assays and surface marker analysis. CFU-GM and BFU-E assays were performed in a semisolid system, and the culture medium consisted of 1% methylcellulose with Iscove's medium supplemented with 30% foetal calf serum, 2% deionized bovine serum albumin, 0.1 mM 2-β-mercaptoethanol, 0.1 mM pyruvate, 2 mM L-alanyl-L-glutamine and 1% penicillin–streptomycin B. Cells were plated in triplicate 1 ml cultures at 10³ cells/ml in Petri dishes with SCF, GM-CSF, G-CSF, IL-3 (10 ng/ml) and incubated at 37°C in a humidified 5% CO₂ atmosphere until *in situ* scoring on an inverted microscope on d14. The pattern of differentiation of the CD34⁺ cells during liquid culture was determined using directly conjugated anti-CD38 (HIT2), anti-CD13 (SJ1D1), anti-CD14 (TUK4), anti-CD15 (C3D-1), anti-CD36 (CLB-IVC7) and anti-glycophorin A (JC159) mAbs.

In this study, ATRA induced the upregulation of CD38 on CD34⁺ cells was verification of ATRA activity (Figure 1c).⁸ As presented in Figure 1a, the number of cells increased during liquid culture without ATRA ($P=0.011$), and the number of viable CD34⁺ cells was not significantly modified. Interestingly, ATRA-exposure over 7 days did not affect the total number of d7 cells, whereas the number of CD34⁺ cells

Figure 1 Effects of pharmacological doses of ATRA on normal marrow CD34 + cells cultured for 7d in a liquid medium supplemented with IL-3, IL-6 and SCF. To evaluate the effects of ATRA, comparisons were performed using Wilcoxon's test and data were considered statistically significant if P -values were <0.05 (*). **(a)** Selected CD34 + cells were exposed to ATRA (1 μ M) from d0 to d7. The number of d7-viable cells are expressed as the percentage of d0 cells. **(b)** High-resolution cell cycle analysis of CD34 + cells. Analysis was performed at d0, d3 and d7. ATRA was added at d0 or d3. Cells were stained with anti Ki67-FITC, anti-CD34-PE mAbs and 7-AAD, and percentages of G0, G1 and S + G2/M cells were determined by flow cytometry. **(c)** Clonogenic assays (left) and phenotypic analysis (right) of d7 cells. The numbers of CFU-GM and BFU-E were quantified before and after ATRA exposure and the results are expressed as percentages of d0-clonogenic progenitors. CD38, CD36 and CD13 expression was evaluated on d7-CD34 + cells and glycophorin A (GlyA), CD15 and CD14 expression on d7-CD34- cells

was lower ($P=0.012$). This could be explained by blocking of CD34 + cells in G1 phase of the cell cycle and induction of differentiation. Study of the cell cycle status of CD34 + cells revealed that ATRA promotes G1 commitment of G0-CD34 + cells ($26.2 \pm 3.0\%$ of d0-CD34 + cells), the effect being reinforced after short (3 days) liquid culture without ATRA. Moreover, we did not observe any concomitant increase in S + /G2/M-CD34 + cells, evidence of the arrest of CD34 +

cells in G1 phase of the cell cycle (Figure 1b). As expected, ATRA also induced differentiation of CD34 + cells in the granulocytic lineage, d7-CD34 + CD13 + and d7-CD34-CD15 + cell numbers being increased ($P=0.043$). Conversely, monocytic and erythroid differentiation pathways were inhibited, as demonstrated by a decrease in CD14 + and GlyA + cells in d7-CD34- cells ($P=0.012$ and 0.027 , respectively), a decrease in d7-CD34 + CD36 + cells

($P=0.018$) and a decrease in the clonogenicity of BFU-E ($P=0.028$) (Figure 1c).

Our experiments focused on the *in vitro* effects of supraphysiological concentrations (micromolar range) of ATRA, which are the levels attained in the plasma of patients treated with ATRA, particularly for MDS. The interesting effects of ATRA on the differentiation of the abnormal clone could be thwarted by deleterious consequences on normal residual CD34+ cells by at least two effects, that is, antiproliferative action and inhibition of erythropoiesis. In this study, the antiproliferative action *in vitro* of ATRA was clearly demonstrated by the decrease in CD34+ cell count and the blocking in G1 phase of the cell cycle, which could be explained by the overexpression of p27^{Kip1}, as previously reported with myeloid cell lines.⁹ Moreover, while granulocytic differentiation was promoted as expected, there was a concomitant inhibition of normal erythropoiesis, in accordance with the ATRA-induced downregulation of GATA-1 observed in marrow CD34+ cells.¹⁰ These results could in part explain the disappointing results of retinoid therapy for MDS.

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