Brief Definitive Report

All-*Trans*- and 9-*Cis*-Retinoic Acid: Potent Direct Inhibitors of Primitive Murine Hematopoietic Progenitors In Vitro

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Summary

Retinoic acid (RA) stimulates the clonal proliferation of mature bone marrow progenitor cells and inhibits the growth of leukemic progenitors, whereas its effects on normal primitive hematopoietic progenitors have not yet been investigated. This study investigated the ability of all-*trans*- and 9-*cis*-RA to modulate the proliferation and differentiation of murine $Lin^-Sca\text{-}1^+$ bone marrow progenitor cells. Both RA isoforms inhibited in a reversible and dose-dependent fashion, the proliferation of multi- but not single-factor responsive $Lin^-Sca\text{-}1^+$ progenitor cells. The 50% effective dose was 10 nM for both all-*trans*- and 9-*cis*-RA. Maximum inhibition was observed at 100–1,000 nM RA, resulting in a 50–75% reduction in the number of proliferative clones. $Lin^-Sca\text{-}1^+$ cells with high proliferative potential were preferentially inhibited by RA, resulting in a 80–100% inhibition depending on the hematopoietic growth factors stimulating their growth. The inhibitory effects of RA were directly mediated on the target cell, since the effects were observed at the single cell level. Furthermore, autocrine transforming growth factor β (TGF-β) production can probably not account for the observed inhibitory effects of RA, since a TGF-β neutralizing antibody did not block RA-induced inhibition. Whereas RA, in general, is a differentiation-inducing agent, treatment of $Lin^-Sca\text{-}1^+$ progenitors resulted in the accumulation of an increased fraction of blasts and immature myeloid cells. Thus, RA inhibits the proliferation as well as differentiation of normal primitive hematopoietic progenitor cells.

The proliferation and differentiation of pluripotent and committed hematopoietic progenitor cells is controlled by a complex network of cytokines and cell to cell interactions (1–5). Retinoic acid (RA) is an oxidized derivate of retinol demonstrated to play an important role in the growth and differentiation of a number of cell types, including epithelial and hematopoietic cells (6–8). In contrast to cytokines, vitamin A cannot be synthesized endogenously, and is therefore considered an essential nutrient (9). RA has been demonstrated to induce granulocytic differentiation of HL-60, a human promyelocytic leukemia cell line, as well as fresh acute promyelocytic leukemia cells (APL) (10, 11), resulting in complete remissions in APL patients (12, 13). Whereas all-trans-RA mediates its effects through the RA receptors (RARs) (14, 15), another RA isoform, 9-*cis*-RA, signals through both the RARs as well as another group of transcription factors, the retinoid X receptors (RXRs) (16).

A striking feature of RA is its apparent opposing actions on the growth of normal and malignant myeloid progenitor cells, in that it stimulates the myeloid growth of normal bone marrow progenitor cells (7, 8, 17–21), whereas it inhibits the proliferation of acute myelogenous leukemia cells in vitro (22).

Previous studies have only examined the effects of RA on unfractionated bone marrow cells, where committed progenitor cells dominate. Thus, potential effects of RA on primitive progenitor cells remain undetermined. In this study we therefore examined the direct effects of escalating concentrations of all-*trans* and 9-*cis* RA on murine $Lin^-Sca\text{-}1^+$ bone marrow cells, previously shown to be highly enriched in primitive hematopoietic progenitor cells (23, 24), to possess high radioprotective efficiency, and capability of long-term reconstitution of all cell lineages in the blood (23, 24).

Materials and Methods

RA, Hematopoietic Growth Factors, and Antibodies. All-*trans*-RA was purchased from Sigma Chemical Co. (St. Louis, MO), whereas 9-*cis*-RA was a gift from Hoffmann-La Roche (Basel, Switzerland). Purified recombinant human (rHu) G-CSF, recombinant murine (rMu)GM-CSF, and recombinant rat (rr) stem cell factor (SCF) were generously supplied by Drs. Ian K. McNiece, Thomas C. Boone, and Keith E. Langley (Amgen Corp., Thousand Oaks, CA).

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rMuIL-3 was purchased from Peprotech Inc. (Rocky Hill, NJ). rHuCSF-1 was kindly provided by Dr. Michael Geier (Cetus Corp., Emeryville, CA). rHuIL-6 was a generous gift from Genetics Institute (Cambridge, MA), purified rHuIL-1α was kindly supplied by Hoffmann-La Roche, and rHuTGF-β1 was provided by Tony Purchio (Oncogene Corp., Seattle, WA). Unless otherwise indicated, all growth factors were used at predetermined optimal concentrations: rMuGM-CSF, 20 ng/ml; rHuG-CSF, 20 ng/ml; rMu IL-3, 20 ng/ml; rHuCSF-1, 50 ng/ml; rrSCF, 100 ng/ml; rHuIL-1α, 20 ng/ml; rHuIL-6, 50 ng/ml; and rHuTGF-β1, 2 ng/ml.

A neutralizing mouse IgG1 antibody against TGF-β1 and TGF-β2 was a gift from Dr. James R. Dash (Celtrix Laboratories, Palo Alto, CA). This antibody neutralizes the effects of mouse as well as human TGF-β1 and TGF-β2 (25, 26). An isotype-matched irrelevant antibody was used as a control.

Isolation of Lin$^-$Sca-1$^+$ Bone Marrow Cells. Lin$^-$Sca-1$^+$ cells were isolated from C57BL6 mice as previously described (24, 27). Briefly, 4–6 $\times$ 10^7 Lin$^-$ cells (27) were resuspended per milliliter of complete IMDM and incubated for 30 min on ice with either FITC-conjugated rat anti-mouse Ly-6A/E antibody (Pharmingen, San Diego, CA) or an isotype-matched control antibody. The cells were washed twice, and Lin$^-$Sca-1$^+$ cells sorted on a cell sorter (Epics Elite; Coulter Electronics, Hialeah, FL). The final recovery of Lin$^-$Sca-1$^+$ cells was 0.05–0.1% of the unfractionated bone marrow. In agreement with others (24), as few as 100 of these cells could protect 50% of lethally irradiated mice (data not shown).

Colony Formation in Semisolid Medium. A modification of the method described by Stanley et al. (28) was used to measure colony formation of murine bone marrow progenitors in vitro. Lin$^-$Sca-1$^+$ bone marrow cells were plated in complete IMDM and 0.3% Seaplaque agarose (FMC Bioproducts, Rockland, ME) and incubated at 37°C in 5% CO_2 for 7 d and scored for colony growth (>50 cells).

High Proliferative Potential Colony-forming Cell (HPP-CFC) Assay. The HPP-CFC assay was performed as previously described by others (29). Alpha modification of Eagle's MEM (MEM-Alpha, GIBCO BRL, Gaithersburg, MD) supplemented with 20% FCS (Sera Lab, Sussex, UK) and L-glutamine was used in all HPP-CFC assays. Cytokines were incorporated into a 1-ml underlayer at a maximum of 3% of the culture volume, and 600 Lin$^-$Sca-1$^+$ cells were incorporated into a 0.5-ml overlayer. The final concentration of Seaplaque agarose was 0.5% in the underlayer and 0.3% in the overlayer. HPP-CFC colonies were scored as dense colonies with a diameter of >0.5 mm after 12–14 d of incubation at 37°C, 5% O_2, 10% CO_2, and 85% N_2.

Single Cell Proliferation Assay. Lin$^-$Sca-1$^+$ were seeded in Terasaki plates (Nunc, Kamstrup, Denmark) at a concentration of one cell per well in a volume of 20 μl complete IMDM. Wells were scored for colony growth (>50 cells) and clusters (10–50 cells) after 12 d of incubation at 37°C and 5% CO_2 in air.

Cell Phenotyping. Lin$^-$Sca-1$^+$ cells were plated in complete IMDM and incubated for 12–14 d with predetermined optimal concentrations of cytokines and RA at 37°C and 5% CO_2 in air. Cell morphology was determined following Giemsa staining of cytospin preparations.

Results and Discussion

To investigate the ability of RA to affect the proliferation of primitive progenitor cells, single Lin$^-$Sca-1$^+$ cells were stimulated by rrSCF and rHuIL-6 in the absence or presence of increasing concentrations of all-*trans*- or 9-*cis*-RA (Fig. 1). In light of most previous studies suggesting that RA stimulates the proliferation of normal myeloid bone marrow progenitor cells (7, 8, 17–21), it was surprising that both all-*trans*- and 9-*cis*-RA inhibited Lin$^-$Sca-1$^+$ colony formation in a concentration-dependent manner. The 50% effective dose (ED_{50}) was ~10 nM for both all-*trans*- and 9-*cis*-RA (Fig. 1). Furthermore, all-*trans*- and 9-*cis*-RA showed comparable concentration–response curves, resulting in a >80% inhibition of colony formation at 100–1,000 nM all-*trans*- or 9-*cis*-RA (Fig. 1). These inhibitory concentrations of all-*trans*- and 9-*cis*-RA are comparable with concentrations of retinoids previously shown to be inhibitory on leukemic hematopoiesis in vitro (17, 19, 22). Furthermore, the inhibitory effects of 9-*cis*-RA and all-*trans*-RA were completely reversible after exposure of Lin$^-$Sca-1$^+$ cells to 1,000 nM of either isoform for 12–24 h (data not shown).

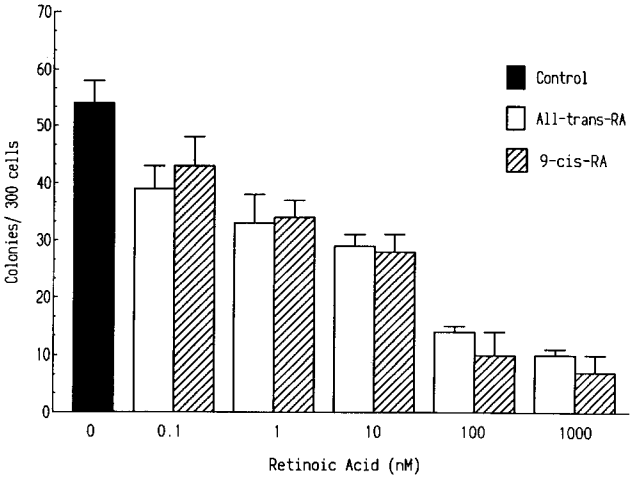

Figure 1. Dose–response of all-*trans*- and 9-*cis*-RA on proliferation of single Lin$^-$Sca-1$^+$ progenitor cells. Lin$^-$Sca-1$^+$ cells were plated at a density of one cell per well in microtiter wells in complete IMDM supplemented with SCF (100 ng/ml) and IL-6 (50 ng/ml), and exposed to increasing concentrations of all-*trans*- or 9-*cis*-RA as indicated. Wells were scored for growth (>10 cells) after a 12–14-d incubation at 37°C and 5% CO_2 in air. Results are the mean (+ SEM) of four separate experiments.

Next, the effects of 100 nM 9-*cis*-RA or all-*trans*-RA were investigated on proliferation of single Lin$^-$Sca-1$^+$ cells stimulated by other growth factor combinations (Table 1). As previously reported, the Lin$^-$Sca-1$^+$ progenitors responded poorly to single hematopoietic growth factors (HGFs) except IL-3 (29), and single-factor–induced colony formation was not or only marginally inhibited by both RA isoforms (data not shown). In contrast, a number of two-factor combinations potently stimulating colony formation of Lin$^-$Sca-1$^+$ progenitors were inhibited by both 9-*cis*-RA (Table 1) and all-*trans*-RA (data not shown). Since most primitive hematopoietic progenitor cells in contrast to committed progenitors require more than one HGF to proliferate (28–30), this suggested that RA might preferentially inhibit early hematopoiesis. This was further supported by the observation that whereas the formation of clusters and small colonies was only marginally affected by RA treatment, larger colonies, and in particular those covering >50% of the culture well,

were dramatically and almost completely inhibited by 100 nM 9-*cis*-RA (Table 1) or all-*trans*-RA (data not shown).

Since some heterogeneity has been demonstrated within the Lin$^-$Sca-1$^+$ population of bone marrow cells (30), we next examined the ability of 9-*cis*-RA to inhibit Lin$^-$Sca-1$^+$ HPP-CFCs. The HPP-CFC assay has been shown to detect some of the most immature hematopoietic progenitor cells measurable in vitro, and HPP-CFCs are characterized by the requirement of multiple HGFs for proliferation (29, 31). The formation of HPP-CFC colonies by Lin$^-$Sca-1$^+$ cells stimulated by IL-3 + SCF as well as several three-factor combinations were inhibited 80% or more by 9-*cis*-RA (Fig. 2).

Next, we examined whether the antiproliferative effects of RA were accompanied by effects on differentiation as well. It is interesting to note that when Lin$^-$Sca-1$^+$ were stimulated by SCF + IL-3, preferentially stimulating the production of macrophages, addition of RA resulted in a twofold increase in the relative number of granulocytes and a concomitant 72% reduction in the relative number of macrophages (Table 2). In contrast, when Lin$^-$Sca-1$^+$ were stimulated by SCF + G-CSF, resulting in the formation of predominantly granulocytes, RA did not significantly affect the relative production of granulocytes and macrophages. However, for both combinations, a three- to fourfold increase in the relative number of immature blasts was observed (Table 2, Fig. 3). This suggested that RA might exert its antiproliferative effects, partially by inhibiting myeloid differentiation of Lin$^-$Sca-1$^+$ progenitor cells. This is of particular interest since RA is considered a differentiation inducing agent (9, 10). Thus, RA might inhibit the proliferation as well as differentiation of primitive hematopoietic progenitor cells.

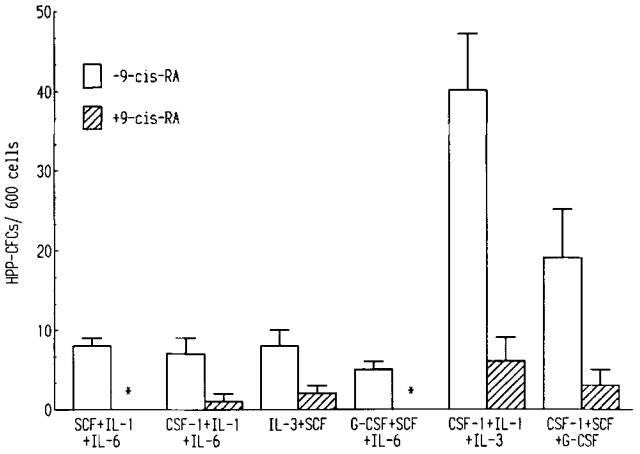

Figure 2. The effect of 9-*cis*-RA on HPP-CFCs. Lin$^-$Sca-1$^+$ cells were isolated and plated (600/plate) into a double-layer agarose culture in the presence of predetermined optimal concentrations of cytokines as described in Materials and Methods. Cultures were scored for HPP-CFC growth after 14 d of incubation in 5% O_2, 10% CO_2, and 85% N_2, at 37°C in the presence or absence of 1,000 nM 9-*cis*-RA. The results represent the mean (+ SEM) of duplicate determinations in at least four separate experiments. (*) No HPP-CFC colonies observed.

RA has been demonstrated to induce TGF-β protein

Table 1. *Effects of 9-cis-RA on Proliferation of Single Lin$^-$Sca-1$^+$ Bone Marrow Progenitor Cells*

Cytokines	9-*cis*-RA	Degree of cell proliferation*				Total clones/ 300 cells
		1	2	3	4	
IL-3 + SCF	–	4	6	10	29	49 (7)
IL-3 + SCF	+	4	4	6	5	19 (3)
IL-3 + IL-6	–	5	10	8	9	32 (5)
IL-3 + IL-6	+	3	6	3	1	13 (2)
IL-3 + GM-CSF	–	4	4	4	7	19 (3)
IL-3 + GM-CSF	+	2	4	2	2	10 (1)
IL-6 + SCF	–	5	11	13	30	59 (7)
IL-6 + SCF	+	4	3	4	1	12 (2)
G-CSF + SCF	–	5	11	9	26	51 (8)
G-CSF + SCF	+	5	8	4	1	18 (3)
CSF-1 + SCF	–	3	3	5	6	17 (3)
CSF-1 + SCF	+	3	2	1	0	6 (1)

Lin$^-$Sca-1$^+$ cells were separated as described in Materials and Methods and plated at one cell per well in 20 μl complete IMDM and predetermined optimal concentrations of cytokines. Cultures were incubated in the presence or absence of 9-*cis*-RA (10^{-7} M) for 12–14 d at 37°C, 5% CO_2 in air, and scored for different degrees of cell growth.

* Scoring criteria 1, 10–50 cells; 2, 50 cells–10% of well covered by cells; 3, 10–50% of well covered; 4, >50% of well covered. Results represent the mean (SEM) of three separate experiments. No proliferation was observed in the presence of medium or 9-*cis*-RA alone.

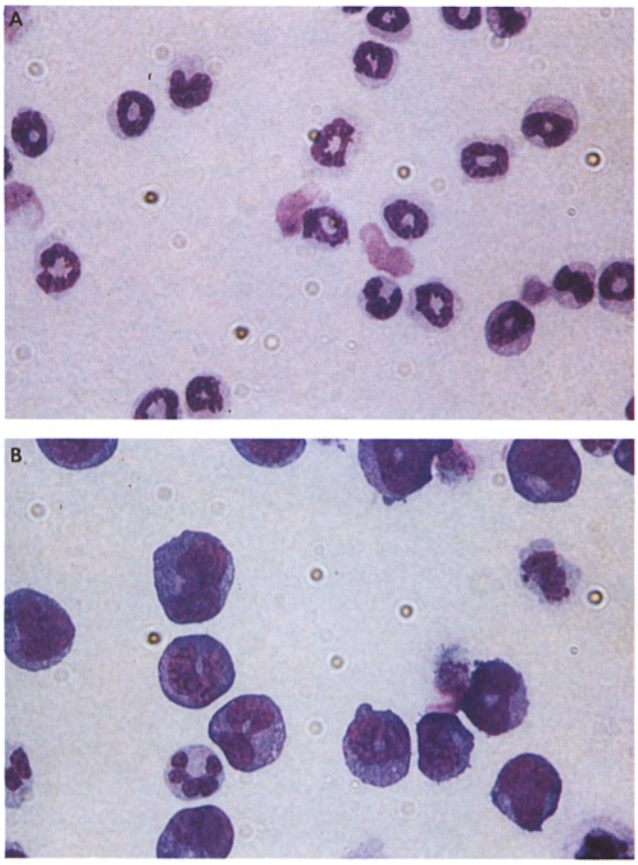

Figure 3. The effects of RA on differentiation of Lin$^-$Sca-1$^+$ bone marrow cells. 500–2,000 Lin$^-$Sca-1$^+$ bone marrow cells were plated in complete IMDM supplemented with predetermined optimal concentrations of G-CSF and SCF in the absence (*A*) or presence (*B*) of 100 nM 9-*cis*-RA. After 12–14 d of incubation at 37°C and 5% CO_2 in air, cytospins were stained with Giemsa. Original magnification at 1,000.

production in a human promyelocytic leukemia cell line (HL-60) (32), and it has also been suggested that normal hematopoietic progenitor cells can produce TGF-β (33). However, in this study the inhibition by 9-*cis*-RA was not significantly affected by a neutralizing TGF-β antibody (Table 3), suggesting that the inhibitory effects of 9-*cis*-RA are not mediated through autocrine TGF-β production. Since this antibody has only been demonstrated to neutralize the effects of TGF-β1 and TGF-β2, it cannot be excluded that TGF-β3, the third TGF-β isoform expressed in mammals, might be involved in the observed RA-induced growth inhibition of Lin$^-$Sca-1$^+$ progenitor cells. However, this seems unlikely for several reasons. First, in the previous study demonstrating autocrine production of TGF-β by hematopoietic progenitor cells, a neutralizing antibody to TGF-β1 could block all the inhibitory response (33). Second, the ability of RA to induce TGF-β production in the HL-60 cell line was exclusively due to enhanced TGF-β1 expression, whereas TGF-β2 and TGF-β3 expression was not affected (32). Thus, although RA might induce TGF-β secretion, these findings suggest that the inhibitory effects of RA on primitive hematopoietic progenitor cells can be mediated through alternative mechanisms.

The present data suggests that all-*trans*- and 9-*cis*-RA are potent inhibitors of proliferation of primitive hematopoietic progenitor cells. Whereas this is in line with what has been previously demonstrated on myeloid leukemic cells (17, 19, 22), it is in contrast to the stimulatory effects shown on normal human bone marrow progenitors (7, 8, 17–21). This apparent discrepancy could be due to the fact that these previous studies have only studied RA effects on unfractionated bone marrow cells. Thus, the observed effects in those studies would be more representative of committed progenitors than the primitive ones investigated here. Proliferative effects on unfractionated bone marrow can also be a consequence of indirect effects through induction of accessory cells to cytokine production. In contrast, RA effects in this study were observed on highly enriched progenitors in single cell experiments, suggesting that the observed inhibitory effects were directly mediated on the progenitors, although autocrine mechanisms cannot be excluded.

Other studies from our laboratory suggest that the effects of RA on CSF-induced proliferation of committed CD34$^+$ human progenitors are strictly dependent on the specific CSF with which it interacts, in that it potently inhibits G-CSF–induced colony formation while it synergizes with GM-CSF (Smeland, E. B., L. S. Rusten, S. E. W. Jacobsen, and

Table 2. *Effects of 9-cis-RA on Differentiation of Lin$^-$Sca-1$^+$ Bone Marrow Cells*

Growth factors	9-*cis*-RA	Percent granulocytes	Percent macrophages	Percent blasts
IL-3 + SCF	–	27 (8)	67 (7)	7 (3))
IL-3 + SCF	+	59 (10)	19 (10)	23 (6)
G-CSF + SCF	–	89 (5)	6 (3)	5 (2)
G-CSF + SCF	+	77 (5)	3 (2)	20 (5)

500–2,000 Lin$^-$Sca-1$^+$ cells were seeded in 0.5–1 ml complete IMDM supplemented with optimal concentrations of cytokines (see Materials and Methods) as indicated, and in the absence or presence of 9-*cis*-RA 100 nM. Cytospin preparations of individual cultures were stained with Giemsa for morphological characterization after 12–14 d of incubation at 37°C and 5% CO_2 in air. Results represent the mean (SEM) of four separate experiments.

Table 3. *Effects of Neutralizing TGF-β Antibody on 9-cis-RA–induced Growth Inhibition of Lin⁻Sca-1⁺ Progenitor Cells*

Stimulation	Anti-TGF-β	Colonies/300 cells
IL-6 + SCF	–	53 (3)
IL-6 + SCF	+	61 (5)
IL-6 + SCF + TGF-β1	–	3 (2)
IL-6 + SCF + TGF-β1	+	48 (7)
IL-6 + SCF + 9-*cis*-RA	–	8 (3)
IL-6 + SCF + 9-*cis*-RA	+	14 (3)

Lin⁻Sca-1⁺ cells were separated as described in the Materials and Methods and plated at a density of one cell per well in 20 μl complete IMDM supplemented with IL-6 (50 ng/ml), SCF (100 ng/ml), TGF-β1 (20 ng/ml), and 9-*cis*-RA (100 nM) as indicated. Cultures were incubated in the absence (–) or presence (+) of 25 μg/ml of a neutralizing anti-TGF-β antibody. An irrelevant control antibody had no effect on colony formation. Colony formation (>10 cells) was scored after 12–14 d of incubation at 37°C and 5% CO_2 in air. The results represent the mean number of colonies (SEM) from four separate experiments, with 300 wells scored per group in each experiment.

H. K. Blomhoff, manuscript in preparation). RA therefore emerges as a pleiotropic regulator of normal hematopoiesis, with potent direct inhibitory effects on primitive progenitors, and bidirectional effects on committed progenitors.

We found all-*trans*- and 9-*cis*-RA to be equipotent inhibitors of Lin⁻Sca-1⁺ progenitor cells. A recent study on human bone marrow cells found the range of activities of the two isoforms to be similar, but 9-*cis*-RA slightly more potent than all-*trans*-RA (19). Whether all-*trans*- and 9-*cis*-RA in fact have differential effects on hematopoiesis will require further studies, including assessments of the relative expression of RARs and RXRs on hematopoietic progenitors.

In conclusion, all-*trans*- and 9-*cis*-RA are potent inhibitors of primitive bone marrow progenitor cells in vitro, and might therefore not have opposing actions on normal and leukemic myeloid progenitor cells as previously suggested.

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