All-Trans Retinoic Acid Directly Inhibits Granulocyte-Colony-Stimulating Factor–Induced Proliferation of CD34+ Human Hematopoietic Progenitor Cells

By Erlend B. Smeland, Leiv Rusten, Stan E.W. Jacobsen, Bjørn Skrede, Rune Blomhoff, Meng Yu Wang, Steinar Funderud, Gunnar Kvalheim, and Heidi Kill Blomhoff

In this study we examine the effects of retinoids on purified CD34+ human hematopoietic progenitor cells. All-trans retinoic acid inhibited granulocyte colony-stimulating factor (G-CSF)–induced proliferation of CD34+ cells in short-term liquid cultures in a dose-dependent fashion with maximal inhibition of 72% at a concentration of retinoic acid of 1 μmol/L. Although no significant effects were observed on granulocyte-macrophage CSF (GM-CSF)–interleukin-3– or stem cell factor (SCF)–induced proliferation, the combinations of G-CSF and each of these cytokines were all inhibited. Moreover, retinol (3 μmol/L) and chylomicron remnant retinyl esters (0.1 μmol/L) in concentrations normally found in human plasma also had inhibitory effects. Single-cell experiments showed that the effects of retinoic acid were directly mediated. Retinoids also significantly inhibited G-CSF–induced colony formation in semisolid medium, with 88% inhibition observed at a concentration of retinoic acid of 1 μmol/L. However, we did not observe any effects of retinoic acid on G-CSF–induced differentiation as assessed by morphology and flowcytometry. Similar to previous findings using total bone marrow mononuclear cells, we observed a stimulation of GM-CSF–induced colony formation after 14 days. We also observed a stimulatory effect of low doses of retinoic acid (30 nmol/L) on blast-cell colony formation on stromal cell layers. Taken together, the data indicate that vitamin A present in human plasma has inhibitory as well as stimulatory effects on myelopoiesis.

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VITAMIN A plays an important role in embryogenesis and in the regulation of growth and differentiation in various cell types. Retinol (ROH) is the major retinoid in plasma, and is transported from liver to extrahepatic target organs via the retinol-binding protein. Newly absorbed retinol is also present as retinyl esters in chylomicrons and their remnants (CMR). After uptake into target cells, retinol can be oxidized to retinoic acid (RA).

Retinoids have been found to exert profound effects on the growth and maturation of hematopoietic cells. Thus, retinoids induce monocytic and/or granulocytic differentiation in various myeloid cell lines. As RA promotes dramatic inhibition of proliferation along with induced differentiation in acute promyelocytic leukemia both in vitro and in vivo, all-trans RA is currently used for therapy in this disease. Interestingly, it has recently been shown that this type of leukemia is characterized by a translocation [t(15;17)] that involves the gene encoding the RA receptor (RAR-α). Conflicting data exist regarding the effects of retinoids on normal hematopoiesis in vitro, although RA has generally been found to stimulate the growth of CFU-GMs. Thus, RA has been suggested to both inhibit granulopoiesis. Because vitamin A can promote a mixture of direct and indirect effects in heterogeneous cell populations like total bone marrow (BM) mononuclear cells, we wanted to examine the effects of retinoids in a more restricted population of purified hematopoietic progenitor cells. In humans, most committed progenitor cells capable of forming colonies in semisolid culture assays have been found to reside in the CD34+ subset of BM cells. The CD34+ population, which represents 1% to 4% of BM mononuclear cells, also includes more primitive hematopoietic progenitor cells. Recent data have shown that enriched CD34+ marrow cells can reconstitute hematopoiesis in vivo in humans and nonhuman primates. In this study we used immunomagnetically purified CD34+ cells to examine the effects of retinoids on enriched populations of hematopoietic progenitor cells. We found that retinoids potently inhibited G-CSF–induced proliferation and colony formation of CD34+ cells. The effect was directly mediated as similar inhibition was observed in single-cell cultures. In addition, physiologic concentrations of retinol and CMR retinyl esters were found to exert similar effects.

MATERIALS AND METHODS

Growth factors and reagents. Purified recombinant human (rHu) granulocyte colony-stimulating factor (G-CSF) and rHu stem cell factor (SCF) were generously supplied by Dr Ian K. McNiece (Amgen Inc, Thousand Oaks, CA). rHu granulocyte-macrophage CSF (GM-CSF) and rHu interleukin-3 (IL-3) were generously provided by Dr Steven Gillis (Immunex Corp, Seattle, WA). Unless otherwise indicated, all growth factors were used at predetermined optimal concentrations: rHuG-CSF (20 ng/mL), rHuGM-CSF (50 ng/mL), rHuIL-3 (20 ng/mL), and rHuSCF (50 ng/mL).

Retinol (Sigma, St Louis, MO) and RA (Sigma) were solubilized in ethanol and kept dark bottled at −20°C. Chylomicron remnants were prepared as described previously. Cells. BM was obtained by iliac crest aspiration from normal adult volunteers with informed consent and the approval of the Ethics Committee of The Norwegian Radium Hospital. BM cells were collected in syringes containing preservative-free heparin (5,000 IU/10 mL BM). Mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation (Lymphoprep; Nyegaard, Oslo, Norway). Positive selection of CD34+ cells was performed as described. Briefly, BM mononuclear cells were resorted with Dynabeads M450
Flow cytometry. Direct immunofluorescence was performed according to standard techniques with phycoerythrin (PE)-labeled HPCA-2 (CD34; Becton Dickinson, San Jose, CA). An isotype-matched, PE-conjugated irrelevant MoAb served as control (Serotec, Oxford, UK). To block unspecific binding via Fc receptors, aggregated human IgG (Dakopatts, Copenhagen, Denmark) was included at a concentration of 100 μg/ml.

Flow cytometric analyses were performed on a FACScan flow cytometer (Becton Dickinson) equipped with an argon-ion laser tuned at 488 nm. Data acquisition and analysis were performed using Lysys II software (Becton Dickinson).

Cell cultures in liquid medium. Cells were grown in triplicates (5 × 10^3 cells/0.2 mL) in round-bottomed microtiter wells (Costar, Cambridge, MA) in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS), penicillin, and streptomycin and different cytokines in the absence or presence of retinoids. Cells were pulsed with 1 μCi of [^3]H-thymidine (The Radiochemical Centre, Amersham, UK) for the last 24 hours of a 6-day incubation, harvested on a Skatron cell harvester (Lier, Norway), and counted on a liquid scintillation counter.

Cell phenotyping. Morphologic analysis of stimulated cells was performed by differential counts on May-Grünwald-Giemsa–stained cytocentrifuge cell preparations.

Colonies in semisolid media. Cells (1 × 10^7 CD34+ cells per tissue-culture grade 35-mm Petri dish) were plated in a volume of 1 mL IMDM (GIBCO, Paisley, UK) supplemented with 20% FCS (Sera-lab, Sussex, UK), 1.2% methylcellulose, 5 × 10^-5 mol/L 2-mercaptoethanol, 300 μg/mL L-glutamine, 66 mg/mL penicillin, 100 mg/mL streptomycin, and recombinant human growth factors as indicated. Colonies (>40 cells) were counted using an inverted microscope after 1 or 2 weeks of incubation at 37°C and 5% CO₂ in air.

Single-cell proliferation assay. CD34+ cells were seeded in Teraski plates (Greiner, Frickenhausen, Germany) at a concentration of 1 cell per well (300 cells per group) in 20 μL IMDM containing 20% FCS, extra L-glutamine, penicillin, and streptomycin. Wells were scored for proliferation (>10 cells) after 2 weeks of incubation at 37°C and 5% CO₂ in air.

Blast colony formation on stromal feeder layers. The blast colony formation on stromal feeder layers. The burst colony cultures were set up essentially as described by Gordon et al. First, feeder layers of stromal cells were prepared by plating 2 × 10^3 BM mononuclear cells in 10 mL of IMDM medium supplemented with 10% FCS, 10% horse serum, and 5 × 10^-3 mol/L hydrocortisone sodium succinate (Sigma; No. H-881) in 50 mL culture tissue flasks. The stromal cultures were fed weekly by complete replacement of the medium, as described above, until a confluent layer of stromal cells covered the base of each dish. In the second step, CD34+ cells (2 × 10^5 cells) in 10 mL of RA containing medium were added to the irradiated stromal layers. After 2 hours of incubation at 37°C in 5% humidified CO₂ in air, unattached cells were removed, and the medium was replaced by 10 mL of 0.3% agar in IMDM medium containing RA and 10% FCS. The number of colonies (>20 cells) with blast cell morphology was counted after 1 week of culture.

RESULTS

Retinoids inhibit G-CSF–induced [^3]H-thymidine incorporation of CD34+ cells in short-term liquid cultures. BM mononuclear cells represent a very heterogeneous cell population allowing a mixture of direct and indirect effects when assessing the effects of biologically active substances. Previous studies on the role of retinoids in hematopoiesis have mostly investigated the effects on unfractionated BM mononuclear cells. Because most progenitors in human BM express the CD34 antigen, we took advantage of a recently developed method for isolation of human CD34+ cells using immunomagnetic beads and an anti-Fab antiserum (DETACHaBEAD) to study the effects of retinoids on a restricted population of immature hematopoietic cells.

We first studied the effects of all-trans RA on CSF- and SCF–induced proliferation of CD34+ cells in liquid cultures (Fig 1). All-trans RA alone had no effects on the [^3]H-thymidine incorporation of CD34+ cells. G-CSF–induced proliferation was inhibited in a dose-dependent way with a 72% inhibition at 1 μmol/L of all-trans RA and significant effects were also observed at physiologic levels (30 nmol/L) of all-trans RA (P < .05). In contrast, all-trans RA did not significantly influence the proliferation induced by IL-3, GM-CSF, or SCF. However, when GM-CSF, IL-3, or SCF were combined with G-CSF, all-trans RA potentely inhibited [^3]H-thymidine incorporation (Fig 2). The RA-mediated inhibition was evident from the onset of the cultures, and cell counting at various time points up to day 14 after stimulation confirmed the inhibitory effects of all-trans RA on G-CSF–induced proliferation of CD34+ cells (data not shown). Furthermore, single CD34+ cell proliferation assays showed that the inhibitory effects of all-trans RA on G-CSF–induced proliferation of CD34+ progenitors were directly mediated (Table 1).

Effect of retinol and retinyl esters bound to chylomicron remnants on mature monocytes. Although effects of all-trans RA on hematopoiesis have been well described, generally retinol...
has been found to have less or no effects on hematopoietic progenitor cells.\(^5\)\(^,\)\(^7\)\(^,\)\(^8\)\(^,\)\(^9\) However, as shown in Table 2, retinol also inhibited G-CSF–induced proliferation in short-term liquid cultures, albeit to a smaller degree than all-trans RA. Moreover, we also wanted to study the effects of retinol bound to its physiologic carriers. As shown in Table 2, physiologic levels of CMR-bound retinyl esters (0.1 pmol/L) also inhibited G-CSF–induced proliferation, again indicating that this is a physiologic effect of retinoids. In contrast, CMR alone did not significantly modulate G-CSF–mediated proliferation at equal concentrations, although CMR alone tended to inhibit proliferation of CD34\(^+\) cells at higher concentrations.

Effects of retinoids on in vitro colony formation. The majority of committed colony-forming cells (CFCs) in BM reside in the CD34\(^+\) population.\(^26\)\(^,\)\(^27\)\(^,\)\(^28\)\(^,\)\(^29\) In agreement with the \(^{[3]}\)H-thymidine incorporation data, we observed a dose-dependent inhibition of G-CSF–induced day 7 colony formation (CFU-G) by all-trans RA (Fig 3). Maximal inhibition of on average 87% was obtained at 1 \(\mu\)mol/L of all-trans RA \((n = 6, P < .05)\). In addition, retinol (3 \(\mu\)mol/L) also significantly inhibited G-CSF–induced colony formation (Fig 3). The effects of all-trans RA were completely reversible after exposure of CD34\(^+\) cells to 100 \(\mu\)mol/L of RA for 24 hours (data not shown). This suggests that the observed inhibition was not caused by toxic effects of RA.

Previous work has established that RA stimulates the formation of CFU-GM colonies.\(^16\)\(^,\)\(^18\)\(^,\)\(^19\)\(^,\)\(^20\)\(^,\)\(^21\) In the present study we found a weak stimulation of day 14 GM-CSF–induced colony growth (Table 3), whereas no distinct colonies were apparent on day 7 on stimulation with rHuGM-CSF alone. In addition, we observed an RA–induced stimulation of day 14 CFU-GM colony formation in response to different growth factor combinations in six of eight experiments, although this did not reach statistical significance \((P = .06, \text{data not shown})\). In contrast, and in agreement with the results presented in Fig 3, G-CSF–induced colony formation was strongly inhibited at day 7 at all concentrations of all-trans RA tested, whereas the effects of RA on G-CSF–induced colony formation at day 14 were less pronounced (Table 3). Although all-trans RA preferentially inhibited G-CSF–induced colony formation at day 7, we also observed significant inhibition of day 14 colony formation in 11 experiments using concentrations of all-trans RA of 1 \(\mu\)mol/L \((P < .001)\) or 100 \(\mu\)mol/L \((P < .05, \text{data not shown})\).

The CD34\(^+\) population also contains, in addition to committed progenitors like CFU-GM, more primitive hematopoietic progenitor cells that are not detected in standard colony assays.\(^28\)\(^,\)\(^29\) Several lines of evidence indicate that cells capa-

![Table 2. Effects of Retinoids and CMR Retinyl Ester on DNA Synthesis of CD34\(^{+}\) Cells in Short-Term Liquid Cultures](image)

Table 2. Effects of Retinoids and CMR Retinyl Ester on DNA Synthesis of CD34\(^{+}\) Cells in Short-Term Liquid Cultures

<table>
<thead>
<tr>
<th>[(^{[3]})H]Thymidine Incorporation (cpm)</th>
<th>Medium</th>
<th>G-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp 1</td>
<td>Exp 2</td>
<td>Exp 1</td>
</tr>
<tr>
<td>No addition</td>
<td>1,709</td>
<td>1,421</td>
</tr>
<tr>
<td>RA (100 nmol/L)</td>
<td>1,123</td>
<td>3,222</td>
</tr>
<tr>
<td>RA (30 nmol/L)</td>
<td>1,087</td>
<td>3,436</td>
</tr>
<tr>
<td>ROH (3 (\mu)mol/L)</td>
<td>1,260</td>
<td>7,251</td>
</tr>
<tr>
<td>CMR-RE (0.1 pmol/L)</td>
<td>233</td>
<td>599</td>
</tr>
<tr>
<td>CMR (0.1 (\mu)mol/L)</td>
<td>2,227</td>
<td>1,221</td>
</tr>
</tbody>
</table>

5 \(\times\) 10\(^4\) CD34\(^{+}\) cells were cultured in triplicate 0.2 mL of medium in microtiter wells and pulsed with \(^{[3]}\)H-thymidine from day 5 to day 6.

![Fig 3. Effects of retinoids on G-CSF–induced colony formation (CFU-G) of CD34\(^{+}\) cells. CD34\(^{+}\) cells were cultured for 7 days in methylcellulose cultures as described in Materials and Methods at 1 \(\times\) 10\(^3\) cells/plate with 20 ng/mL of rHu G-CSF alone or in combination with 30 nmol/L (\(\square\)), 100 nmol/L (\(\square\)) or 1 \(\mu\)mol/L (\(\blacksquare\)) of all-trans RA or 3 \(\mu\)mol/L of retinol (\(\square\)). Results are presented as mean \((\text{SEM})\) number of colonies from six separate experiments with triplicate determinations. *Statistical significance \((P < .05, \text{paired Wilcoxon test})\).
RETINOIDS INHIBIT G-CSF–INDUCED PROLIFERATION

Table 3. Effects of All-Trans RA on G-CSF– and GM-CSF–Induced Day 7 and 14 Colony Formation

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Exp 1 Day 7</th>
<th>Exp 1 Day 14</th>
<th>Exp 2 Day 7</th>
<th>Exp 2 Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF (20 ng/mL)</td>
<td>36 (2.5)</td>
<td>39 (4.0)</td>
<td>39 (4.0)</td>
<td>32 (3.6)</td>
</tr>
<tr>
<td>+RA 30 nmol/L</td>
<td>5 (1.2)</td>
<td>29 (3.5)</td>
<td>6 (0.8)</td>
<td>25 (4.6)</td>
</tr>
<tr>
<td>+RA 1 μmol/L</td>
<td>1 (0.6)</td>
<td>16 (3.8)</td>
<td>1 (1.0)</td>
<td>18 (2.6)</td>
</tr>
<tr>
<td>GM-CSF (60 ng/mL)</td>
<td>0</td>
<td>19 (3.2)</td>
<td>22 (1.2)</td>
<td>0</td>
</tr>
<tr>
<td>+RA 30 nmol/L</td>
<td>0</td>
<td>34 (3.5)</td>
<td>25 (4.0)</td>
<td>0</td>
</tr>
<tr>
<td>+RA 1 μmol/L</td>
<td>0</td>
<td>18 (4.6)</td>
<td>0</td>
<td>29 (5.0)</td>
</tr>
</tbody>
</table>

CD34+ cells (2 × 10^4 cells/dish) were cultured with growth factors in methyl cellulose, as described in Materials and Methods. Data represent mean of triplicates (SD). Two representative experiments are shown.

Table 4. Effects of All-Trans RA on Blast Colony Numbers

<table>
<thead>
<tr>
<th>No. of Colonies/2 × 10^4 CD34+ cells</th>
<th>No addition</th>
<th>+RA (30 nmol/L)</th>
<th>+RA (1 μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>88 (25)</td>
<td>178 (49)*</td>
<td>64 (25)*</td>
</tr>
</tbody>
</table>

CD34+ cells (2 × 10^4) were added to irradiated, preformed stromal layers. After 2 hours of incubation at 37°C in 5% CO₂ humidified atmosphere, unattached cells were removed, and the medium was replaced by 10 mL of 0.3% agar in IMDM medium containing 10% FCS. The number of colonies with blast cell morphology were counted after 1 week of culture. Data represent mean (SEM) of six experiments.

* P < .05, paired Wilcoxon test.

DISCUSSION

Retinoids have been shown to influence growth and differentiation of various hematopoietic cells. However, most studies of the effects of retinoids on normal hematopoiesis have used unfractionated BM mononuclear cells. As retinoids may have indirect effects, for instance via induced secretion of cytokines, it is important to use highly enriched and more homogenous populations of hematopoietic progenitors for such studies. In this study, we have used highly purified CD34+ BM cells, which contain most of the progenitor cells in the BM.

We observed a specific and marked inhibition of G-CSF–induced [3H]-thymidine incorporation and colony formation. The effects of all-trans RA on G-CSF–induced proliferation of CD34+ cells were directly mediated, as all-trans RA also inhibited growth of CD34+ cells in single-cell experiments. G-CSF is known to synergize with several other hematopoietic growth factors, including SCF, and we found that all-trans RA also potently inhibited G-CSF–induced proliferation induced by two-factor combinations of G-CSF and IL-3, GM-CSF, or SCF. Our results are in agreement with the findings of Tohda et al. who showed a specific inhibition by all-trans RA on day 7 colony formation induced by G-CSF using normal BM cells. However, in their study it was not shown whether the effect was directly mediated. In apparent contrast, van Bockstaele et al. found that all-trans RA weakly stimulated the formation of day 14 CFU-G colonies, using isolated CD34+ cells stimulated with a combination of several cytokines, whereas the total number of colonies found, including CFU-M and BFU-E colonies, were significantly depressed. However, it is difficult to directly compare the results of these studies because of the use of different cytokines or cytokine combinations. In addition, whereas we observed the most potent inhibition of G-CSF–induced colony formation at day 7, van Bockstaele et al scored CFU-G colonies only at day 14.

Most available data suggest that retinoids also can stimulate normal hematopoietic progenitor cells. A stimulatory effect of RA on CFU-GM was first shown by Douer and Koeffler, using BM mononuclear cells. This finding has later been confirmed by several groups, and is in general agreement with our results on GM-CSF–induced colony formation of isolated CD34+ cells. Taken together, the data therefore suggest that retinoids can have both stimulatory and inhibitory effects on committed progenitor cells, and that the effects on such cells are strongly dependent on the CSFs stimulating their growth.

Few reports have examined the effects of retinoids on more immature myeloid cells. A few reports have studied the effects of RA on CFU-Mix and no clear effects were found. We found that all-trans RA in low concentrations (30 nmol/L) stimulated blast colony formation on preformed stromal layers, whereas higher concentrations (1 μmol/L) were inhibitory. It is possible that the stimulatory effects observed on blast-cell colony formation in the present study are caused by RA-induced cytokine production in the preformed stromal cell layer. Interestingly, we have recently shown in the murine system that primitive progenitor cells (Lin- Sca-1+) are profoundly and directly inhibited by all-trans RA and 9-cis RA in various assays, including high proliferative potential colony-forming cells colony formation. Similarly, we have recently also found that RA inhibits the growth of CD34+CD38- cells in single cell assays. Whether retinoids also can directly stimulate subpopulations of immature hematopoietic cells will require further studies.
Retinoids are known to induce terminal differentiation of several human and murine myeloid cell lines, especially cell lines expressing features of relatively mature cells (ie, HL-60 and U-937). Moreover, RA has been shown to induce granulocytic differentiation of acute promyelocytic leukemia cells in vivo and in vitro. RA-induced differentiation of cell lines is generally accompanied by reduced proliferation, but inhibited cell growth is not always accompanied by differentiation. However, the effects on differentiation of normal hematopoietic progenitors are controversial. Although the early inhibition of G-CSF-induced growth observed in this study (Table 1) suggests an inhibitory effect by all-trans RA on proliferation, we did not observe any significant effect of all-trans RA on G-CSF-induced differentiation of CD34+ cells in liquid cultures. Although this is in agreement with most previous studies, others have found inhibited granulocytic or increased monocytic differentiation. However, it is difficult to directly compare the results of these studies, as different hematopoietic growth factors were used. Moreover, in most of these studies heterogeneous cell populations were used, and therefore possible effects on differentiation of small subpopulations of the cells could easily be overlooked.

One of the main limitations in most in vitro studies using vitamin A is that the retinoids are administered to the cells solubilized in ethanol or Me2SO, and not bound to its physiological carriers. In vivo, however, retinol is transported in plasma bound to retinol-binding protein (RBP), and is taken up by target cells via RBP-receptors. Newly absorbed retinol is also present as retinyl esters in CMR, which are taken up by cells presumably via binding to low density lipoprotein (LDL) receptors or LDL receptor related protein (LRP). In this study we have observed effects of all-trans RA and retinol in physiological concentrations. Similar effects were also observed using CMR-bound retinyl esters at concentrations of 0.1 μmol/L. Taken together, our experiments therefore suggest that the effects of retinoids on hematopoiesis are physiologically relevant.

**REFERENCES**


| Table 5. Effects of All-Trans RA on G-CSF-Induced Differentiation of CD34+ BM Cells |
|-------------------------------------|-------------------------------------|
|  | Blasts  | Promyelocytes  | Myelocytes  | Metamyelocytes  | Band Granulocytes  | Segmented Neutrophils  | Peroxidase  |
| G-CSF  |  |  |  |  |  |  |  |
| d 3 (1 exp)  | 53  | 23  | 18  | 2  | 4  | 0  | ND  |
| d 6 (3 exp)  | 0  | 5  | 19  | 34  | 37  | 10  | 72  |
| G-CSF + RA  |  |  |  |  |  |  |  |
| d 3 (1 exp)  | 64  | 16  | 6  | 7  | 4  | 3  | ND  |
| d 6 (3 exp)  | 0  | 2  | 12  | 35  | 39  | 5  | 70  |

CD34+ cells were grown in liquid cultures in the presence of G-CSF (100 ng/mL) with or without RA (100 nmol/L). The morphologic evaluation was performed on cytospin specimens stained with May-Grünwald-Giemsa. Figures represent percentages. Freshly isolated CD34+ cells contained >90% cells with blast-cell morphology.


All-trans retinoic acid directly inhibits granulocyte colony-stimulating factor-induced proliferation of CD34+ human hematopoietic progenitor cells

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