Dual Action of Retinoic Acid on Human Embryonic/Fetal Hematopoiesis: Blockade of Primitive Progenitor Proliferation and Shift From Multipotent/Erythroid/Monocytic to Granulocytic Differentiation Program

By Angelo Tocci, Isabella Parolini, Marco Gabbianelli, Ugo Testa, Luisella Luchetti, Paola Samoggia, Barbara Masella, Giovanni Russo, Mauro Valtieri, and Cesare Peschle

In preliminary studies, we have analyzed the hematopoietic growth factor (HGF) requirement of hematopoietic progenitor cells (HPCs) purified from embryonic-fetal liver (FL) and grown in fetal calf serum-supplemented (FCS') clonogenic culture. The key role of erythropoietin (Epo) for colony formation by early erythroid progenitors (burst-forming units-erythroid [BFU-E]) has been confirmed. Furthermore, in the absence of exogenous HGFs, FL monocytic progenitors (colony-forming unit monocyte [CFU-M]) generate large colonies exclusively composed of monocytes-macrophages; these colonies are absent in FCS clonogenic culture. On this basis, we have investigated the role of all-trans retinoic acid (ATRA) and its isomer 9-cis RA in FL hematopoiesis. Both compounds modulate the growth of purified FL HPCs, which show a dose-dependent shift from mixed/erythroid/macrophage to granulocytic colony formation. Studies on unipotent cell culture unequivocally indicate that the shift is mediated by modulation of the HPC differentiation program to the granulopoietic pathway (rather than RA-induced downmodulation of multipotent/erythroid/mesenchymal HPC growth coupled with recruitment of granulocytic HPCs). ATRA and 9-cis RA also exert their effect on the proliferation of primitive HPCs (high-proliferative potential colony-forming cells [HPP-CFCs]) and primitive hematopoietic stem cells (HSCs; assayed in Dexter-type long-term culture). High concentrations of either compound (1) drastically reduced the number of primary HPP-CFC colonies and totally abolished their recloning capacity and (2) inhibited HSC proliferation. It is crucial that these results mirror recent observations indicating that murine adult HPCs transduced with dominant negative ATRA receptor (RAR) gene are immortalized and show a selective blockade of granulocytic differentiation. Altogether, these results suggest that ATRA/9-cis RA may play a key role in FL hematopoiesis via a dual effect hypothetically mediated by interaction with the RAR/RXR heterodimer, ie, inhibition of HSC/primitive HPC proliferation and induction of CFU-GEMM/ BFU-E/CFU-M shift from the multipotent/erythroid/mesenchymal to the granulocytic-neutrophilic differentiation program.

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M B R Y O N I C - F E T A L  L I V E R (FL) cells comprise diverse hematopoietic progenitor cells (HPCs), ie, erythroid (burst-forming unit-erythroid [BFU-E]), granulomonocytic (colony-forming unit-GM [CFU-GM]), and multipotent progenitors (CFU-GEMM), as indicated by in vitro clonogenic assay. In vivo, however, FL HPCs differentiate almost exclusively along the erythroid pathway. Interestingly, the requirement for hematopoietic growth factors (HGFs), also termed interleukins (ILs) or colony-stimulating factors (CSFs), of FL HPCs differs from that of corresponding adult HPCs: thus, FL HPCs stimulated with erythropoietin (Epo) alone under stringent fetal calf serum-free (FCS') culture conditions generate an optimal number of erythroid bursts, which is not significantly enhanced by the combined addition of multilineage HGF(s) (IL-3 and/or GM-CSF). Furthermore, FL HPCs show a higher cycling activity and a shorter doubling time than their adult counterparts, which underlie the rapid expansion of their pool size. All-trans retinoic acid (ATRA) and diverse synthetic analogues affect differentiation of neoplastic and normal hematopoietic cells. In particular, ATRA induces granulocytic differentiation of acute myeloblastic and promyelocytic leukemia (AML and APL) cell lines (eg, HL-60, AML-193, NB4), as well as APL blasts in vivo. A significant role for retinoids in the immunologic system is suggested by the impaired humoral and cellular immune responses in vitamin A-deficient mice. ATRA is also an important morphogen in vertebrate development: (1) in chick embryonal limbs, endogenous ATRA gradients underlie pattern formation and (2) in mouse embryos, perturbation of ATRA concentrations leads to disruptive morphological alterations and homeotic transformations. In vitro ATRA induces differentiation of human teratocarcinoma cells and coordinate expression of homeobox-containing genes.

Previous studies performed on unpurified human and avian bone marrow (BM) HPCs showed a stimulatory and inhibitory effect on granulocytic and erythroid differentiation respectively. However, the possibility could not be excluded that accessory cells coplated with HPCs had modified ATRA metabolism and/or its effect via HGF(s) released in culture. In view of these uncertainties, we analyzed the ATRA action on highly purified HPCs from normal adult peripheral blood (PB). These studies showed that ATRA inhibits erythroid and stimulates granulocytic neutrophilic differentiation, suggesting, but not proving, that ATRA induces an HPC shift from erythroid to granulocytic-neutrophilic differentiation program. Furthermore, this effect was coupled with and possibly mediated by downmodulation of the erythroid transcription factors GATA-1 and NF-E2.

In this study, we have analyzed (1) the HGF requirement of purified FL HPCs in FCS clonogenic culture, including primitive HPCs (high proliferative potential colony-forming cells [HPP-CFCs]); (2) the effects of ATRA and its isomer 9-cis RA on HPC proliferative and differentiative programs

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and clonogenic cultures, including paired daughter cell analysis; and (3) the effects of ATRA and 9-cis RA at the level of primitive HPCs (HPP-CFC assay) and putative hematopoietic stem cells (HSCs; as evaluated by the number of HPCs generated after 5 weeks in Dexter-type long-term culture (LTC)).

**MATERIALS AND METHODS**

**HGFs and Chemical Inducers**

The following recombiant human HGFs were used: IL-3 and GM-CSF (1.7 to 2.5 x 10^6 U/mg; The Genetics Institute, Cambridge, MA); Epo and e-kit ligand (KL; Amgen, Thousand Oaks, CA, and Immunix, Seattle, WA); and G-CSF and M-CSF (CSF- D Systems, Minneapolis, MN). ATRA was obtained from Sigma (St Louis, MO), and 9-cis RA and vitamin D3 (1a-25-OH-VitD3) were from Roche Co (Basel, Switzerland).

**Cell Purification and Phenotype Analysis**

FL samples were obtained from spontaneously aborted fetuses (6 to 10 weeks gestation), after informed consent of the mothers, and dissected within 3 hours of delivery. A single-cell suspension was prepared by serial passage through 19-, 21-, and 22.5-gauge needles. Cells were washed and diluted in FCS culture medium containing Iscove’s modified Dulbecco’s medium (IMDM; Hyclone, Logan, UT), bovine serum albumin (BSA; 10 mg/mL), pure human transferrin (1 mg/mL), human low-density lipoprotein (40 mg/dL), insulin (10 µg/mL), sodium pyruvate (10^{-4} mol/L), L-glutamine (2 x 10^{-4} mol/L), rare inorganic elements supplemented with saturated levels of all from Sigma). Cells (10^6 cells/mL) were incubated overnight in 12.5 mmakers (Falcon; Becton Dickinson BD, Oxnard, CA) at 37°C in a 5% CO2/5% O2/90% N2 humidified atmosphere. The suspension was then centrifuged at 100g for 10 minutes to separate hepatocytes from hematopoietic cells. Cell viability was always higher than 95% as assessed by trypan-blue dye exclusion test. Two different protocols were used for HPC enrichment.

**Protocol A.** Cell suspension was fractionated by centrifugation through a Ficoll density gradient (d = 1.077); the resulting cell suspension was washed twice and labelled with phycoerythrin (PE)-conjugated anti-CD34 monoclonal antibody (MoAb; anti-HPCA2, Becton Dickinson, Mountain View, CA). The labeled cell suspension was sorted by FACScan (Becton Dickinson).

**Protocol B.** Cell suspension was fractionated by centrifugation through a Percoll density gradient (d = 1.080). CD34^+ cells were further separated by a magnetic cell separator (MACS; Miltenyi Biotech, Sunnyvale, CA) and the MACS CD34 isolation kit, essentially composed by a modified anti-CD34 MoAb QBend10 (anti-HPCA1 epitope; mouse IgG1, reagent A) and colloidal superparamagnetic microbeads recognizing the anti-CD34 MoAb (reagent B). Briefly, 20 µL of reagent A was added to 10^7 nucleated cells and incubated for 15 minutes in phosphate-buffered saline (PBS) with 2% BSA. Cells were washed once and labeled with reagent B. After 15 minutes at 4°C, cells were separated. Columns introduced in a magnetic field were washed with PBS and 2% BSA. Labeled cells were applied, and unbound cells were washed out with 1.5 mL of PBS/BSA. Columns were removed from the magnetic field and the bound cells were eluted. This procedure was repeated twice. Eluted cells were collected in a sterile tube and counted in a hemocytometer.

**ATRA Effects in Unicellular Culture of HPCs and HPC-Derived Paired Daughter Cells**

Unicellular cultures were performed in flat-bottomed 96-microplate wells in 0.1 mL of FCS medium supplemented with saturating levels of all HGFs supplemented in semisolid culture (see above) combined or not with ATRA (10^{-9} mol/L). Cells were seeded at 0.5 cell/well density. After 10 to 12 days, single colonies were scored, picked up, and smeared on glass slides for morphology analysis.

In the paired sibling cell assay, 0.5 cell/well was plated in 0.1 mL of FCS medium containing saturating levels of HGFs as indicated above. At day 1 to 2, paired sibling cells were identified, picked up by a micromanipulator, and seeded in two sibling wells containing 0.1 mL of the same medium treated or not with ATRA (10^{-9} mol/L). After 10 to 12 days, colonies were scored and analyzed as described above.
HPP-CFC Assay

The frequency of HPP-CFCs was evaluated as previously described. Briefly, 100 enriched HPCs (purification protocol B) were added to 1 mL of culture medium composed of 40% FCS and 0.9% methylcellulose in IMDM supplemented with α-thioglycerol (10-4 mol/L), pure human transferrin (1 mg/mL) and ferric ammonium citrate (10 μg/mL). The following HGFs were added: KL (100 ng/mL), IL-3 (100 U/mL), GM-CSF (10 ng/mL), G-CSF (500 U/mL), M-CSF (500 U/mL), Epo (3 U/mL). Cells were incubated at 37°C in a fully humidified atmosphere at 33°C. After formation of a confluent layer (mean ± standard error of mean [SEM] values from three separate representative experiments), Fluorescence-activated cell sorter (FACS) analysis of a representative protocol B experiment is shown in Fig 1. Both methodologies provided a similar HPC frequency (range, 30% to 80%, see Figs 2 through 5, Tables 1 and 2), but protocol B allowed a more abundant HPC yield (51.8% ± 29.5% for protocol B vs A, as evaluated with respect to HPC number in the Ficoll fraction).

Using protocol A, clonogenic experiments were performed to identify the optimal HGF combination for FL HPC colony growth in FCS* semisolid culture (Fig 2). FCS alone supported formation of only CFU-M colonies: the monocyte-macrophage colony composition was confirmed not only by morphology, but also anti-CD14 immunoperoxidase analysis (using the latter technique, granulocytic cells are only slightly labeled, while monocytes-macrophages are intensively positive). Epo addition stimulated BFU-E colony formation (see also Valtieri et al.), but did not significantly modify the number of CFU-M colonies. Further addition of IL-3 and GM-CSF mildly increased the number of BFU-E and CFU-M colonies, but markedly enhanced their colony size (results not shown), as reported in Valtieri et al; this HGF combination also induced pure CFU-G colonies and CFU-GM clones with prevalent monocytic content, which were scored as CFU-M clones (Figs 2 through 5 and text; see also below). KL added together with Epo, IL-3, and GM-CSF moderately stimulated CFU-GEMM colony formation and increased the size of all other colony types (not shown), but did not significantly modify the total number of colonies. Thus, optimal growth of enriched HPCs was demonstrated in the presence of KL (100 or 10 ng/mL), IL-3 (100 U), GM-CSF (10 ng), and Epo (3 U) (Figs 2 and 3), as confirmed with HPCs purified using protocol B (Figs 4 and 5).

Enrichment and HGF Requirements of FL HPCs

Enrichment level, as assessed by phenotypic reanalysis of the CD34 antigen, was comparable for both protocols A and B (85.7 ± 3.6 and 86.8 ± 3.4 CD34* cells respectively; mean ± standard error of mean [SEM] values from three separate representative experiments). Fluorescence-activated cell sorter (FACS) analysis of a representative protocol B experiment is shown in Fig 1. Both methodologies provided a similar HPC frequency (range, 30% to 80%, see Figs 2 through 5, Tables 1 and 2), but protocol B allowed a more abundant HPC yield (51.8% ± 29.5% for protocol B vs A, as evaluated with respect to HPC number in the Ficoll fraction).

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Dexter-Type LTC

Methodology for Dexter-type LTC has been modified as follows. Briefly, the BM aspirate Ficoll-Hypaque cut was inoculated with ATRA or 9-cis RA. The cultures were terminated by enzymatic digestion and enrichment level, as assessed by phenotypic reanalysis of the CD34 antigen, was comparable for both protocols A and B (85.7 ± 3.6 and 86.8 ± 3.4 CD34* cells respectively; mean ± standard error of mean [SEM] values from three separate representative experiments). Fluorescence-activated cell sorter (FACS) analysis of a representative protocol B experiment is shown in Fig 1. Both methodologies provided a similar HPC frequency (range, 30% to 80%, see Figs 2 through 5, Tables 1 and 2), but protocol B allowed a more abundant HPC yield (51.8% ± 29.5% for protocol B vs A, as evaluated with respect to HPC number in the Ficoll fraction).

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Effect of ATRA and 9-cis RA on Purified FL HPCs in Clonogenic Cultures

Preliminary ATRA dose/response experiments on FL HPCs purified by protocol A and grown in semisolid culture indicated that the saturating ATRA dosage was 10-7 to 10-6 mol/L (results not shown, see also below). Figure 2 shows the effects of diverse combinations of HGFs at saturating levels and ATRA (10-7 mol/L) in purified FL HPC clonogenic culture: (1) in absence of exogenous HGFs, addition of ATRA totally abolished CFU-M colony formation; (2) in the presence of Epo alone, both BFU-E and CFU-M colonies were suppressed; (3) when multilineage HGFs were supplemented, ATRA dramatically increased the number of CFU-G colonies (these included a minority [<1 of 3] of CFU-GM clones composed of ~85% granulocytic cells, which were thus scored as CFU-G colonies), while inhibiting formation of both BFU-E (including a few CFU-GEMM) and CFU-M colonies (the latter comprised a minority [<1 of 3] of CFU-GM clones containing ~70% to 75% monocytic cells, which were thus scored as CFU-M colonies); (4) similarly, in experiments with IL-3, GM-CSF, and KL, addition of ATRA sharply increased the number of CFU-G colonies and inhibited that of both BFU-E/CFU-GEMM and CFU-M (the incidence and composition of CFU-GM clones was as in [3]); (5) it is also noteworthy that the ATRA-induced inhibition of BFU-E and CFU-M growth was dose-dependent (Table 1, see also below).

In selected experiments, colonies were individually picked up, smeared onto glass slides, stained, and cell composition analyzed. As expected, CFU-G and CFU-M colonies were entirely composed of granulocytic and monocytic cells, respectively. In a representative experiment, control CFU-GM colonies (n = 6) comprised 86% ± 5.1% (mean ± SEM) monocytic and 14% ± 5.1% granulocytic cells, whereas CFU-GM colonies (n = 6) treated with 10-6 mol/L ATRA were composed of 25% ± 4.6% monocytic and 75% ± 4.6% granulocytic cells. Similar results were obtained picking up CFU-GM colonies from duplicate dishes (data not shown). This cell composition pattern was confirmed in unicellular HPC cultures: In a representative experiment, control CFU-
GM clones ($n = 5$) were composed of $72\% \pm 4.3\%$ monocytic and $28\% \pm 4.3\%$ granulocytic cells, while in ATRA-treated cultures, CFU-GM colonies ($n = 10$) comprised $29\% \pm 5.1\%$ monocytic and $71\% \pm 5.1\%$ granulocytic cells.

To assess the level at which ATRA exerted its effects, ATRA ($10^{-6}$ mol/L) was added either on day 0 or with a delay of 2, 4, 6, or 8 days to clonogenic cultures performed under optimal growth conditions (Fig 3). Day 0 addition of ATRA induced a significant modulation of FL HPC proliferation/differentiation, whereas delayed ATRA addition had little or no effect at early or late culture days respectively, thus suggesting that the ATRA effect is mainly exerted on HPCs at initial stages of HPC differentiation. Here again, CFU-G and CFU-M colonies comprised $<30\%$ CFU-GM clones mainly composed of granulocytic cells (scored as CFU-G colonies) and monocytic cells (scored as CFU-M colonies), respectively.

Results obtained on HPCs enriched by protocol A were confirmed and extended to 9-cis RA by using the HPCs purified by protocol B (Figs 4 and 5). Again, (1) addition
Fig 2. Cloning of purified FL HPCs (100 cell/dish) in FCS* semisolid medium containing Epo (3 U/mL), IL-3 (100 U), GM-CSF (10 ng), KL (100 ng), ATRA (10^-7 mol/L) in various combinations as indicated. CFU-GEMM colonies present in KL-treated cultures were cumulated with the prevailing BFU-E colonies. Few CFU-GM colonies were present (see Results): they comprised 85% monocytic cells in the absence of ATRA and were scored as "CFU-M," while they were composed of 70% to 75% granulocytic cells when ATRA was added and were scored as "CFU-G" (see Results). Mean ± SEM values from seven separate experiments, each in duplicate dishes. *P < .02. **P < .001 when comparing corresponding ATRA+ versus ATRA- cultures. FL HPCs were purified using method A.

of ATRA or 9-cis RA markedly reduced the number of BFU-E/CFU-GEMM and CFU-M clones and significantly increased the number of CFU-G colonies under optimal culture conditions; (2) this effect was dose-dependent and fully exerted starting at 10^-7 mol/L for both retinoids; (3) neither retinoid significantly modified the total number of colonies. The frequency of CFU-GM colonies predominantly granulocytic (scored as CFU-G) and predominantly monocytic (scored as CFU-M) was similar to that observed in the experiments described above.

Effect of ATRA on HPC Unicellular Culture

To avoid the bias of coplated accessory cells, we added 10^-6 mol/L ATRA to unicellular FCS* culture (0.5 cells/well), stimulated by saturating levels of KL, IL-3, GM-CSF, and Epo. In a representative experiment (Table 2) with 80% cloning efficiency, ATRA dramatically reduced the number of BFU-E and CFU-M colonies and conversely increased the number of CFU-G colonies. The incidence of CFU-GM colonies predominantly granulocytic (scored as CFU-G) and monocytic (scored as CFU-M) was similar to that in the above described experiments.

Effect of ATRA on Paired Daughter Cell Culture

In a final series of studies, we analyzed the effects of ATRA on paired daughter cells separately grown in unicellular cultures (0.5 cells/well) using an optimal HGF stimulus (Table 3 shows the cumulative results of three independent experiments). (1) Control experiments: to assess the possibility of asymmetric divisions, control cultures were performed with no ATRA addition in the two sibling wells. Under these experimental conditions, asymmetric divisions were not observed (except for a mixed v monocytic colony); (2) ATRA treatment studies: ATRA- sibling wells mostly contained erythroid or monocytic colonies; a few wells comprised mixed, granulomonocytic, or granulocytic clones (predominantly eosinophilic); other ones contained abortive colonies, which hypothetically had undergone apoptosis. Strikingly, ATRA+ sibling wells always contained either granulocytic (predominantly neutrophilic) or abortive colonies: particularly, ATRA- erythroid, monocytic, granulomonocytic or mixed sibling colonies corresponded to ATRA+ granulocytic or abortive sibling colonies.

Effect of ATRA and 9-cis RA on Purified FL HPP-CFCs

To further clarify the level at which ATRA exerts its effects, we analyzed the effect of ATRA treatment on primitive HPCs defined as HPP-CFCs (Fig 6). Experiments performed upon addition of saturating amounts of KL, IL-3, GM-CSF, G-CSF, M-CSF, Epo to FCS* culture medium demonstrated that (1) the majority of colonies morphologically and functionally defined as HPP-CFC-derived clones (ie, >.05 mm in diameter and with recloning potential) displayed a pure monocytic morphology, while (2) a minority displayed a granulomonocytic composition. (3) It is also noteworthy that few primary colonies of large size and erythroid or granulocytic morphology were observed: these col-
cones gave rise to only a few clusters in secondary culture and, therefore, were not considered as HPP-CFC-derived. Starting at the $10^{-7}$ mol/L dose, ATRA drastically reduced the number of primary HPP-CFC colonies and totally abolished their cloning capability (Fig 6). Similar results were obtained with 9-cis RA (not shown).

**Effect of ATRA and 9-cis RA on FL Dexter-Type LTC**

In 5- to 8-week adult LTC, the number of HPCs is directly related to the number of LTC-initiating cells (LTC-ICs), which are considered to represent putative HSCs. Both ATRA and 9-cis RA drastically reduced the number of HPCs in 5-week FL LTC suspension and adherent fractions. In a representative experiment (Fig 7), we scored 71.5 colonies in the control culture (70.0 in the adherent layer), 13.0 colonies in the ATRA-treated cultures (all in the adherent layer), and 8.0 in the 9-cis cultures (7.0 in the adherent layer). These colonies were granulomonocytic by morphological analysis, with prevalent monocytic or granulocytic cells in ATRA- or ATRA+ cultures, respectively.

**DISCUSSION**

The effect of ATRA and 9-cis RA was evaluated on enriched FL HPCs: the purification protocol, initially based on flow cytometry sorting, was improved by a modified magnetic separation protocol for more abundant HPC yield. The HGF requirement for optimal FL HPC growth was investigated. CD34+ HPCs generated BFU-E colonies in FCS+ semisolid cultures supplemented with Epo only: this observation confirms that Epo alone induces FL BFU-E colony formation, whereas it has little effect by itself on adult BFU-E.

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**Fig 3.** Time-course effect of ATRA (10^{-6} mol/L) on purified FL HPCs cloned in FCS+ semisolid medium supplemented with Epo, IL-3, GM-CSF, KL (see legend to Fig 2). ATRA concentrations and addition times are indicated. Few CFU-GM colonies were scored (see Results). ATRA addition caused a time-related, gradual switch of CFU-GM clones from a predominantly monocytic to a mainly granulocytic cell composition (CFU-GM colonies predominantly monocytic or granulocytic were scored as CFU-M and CFU-G, respectively; see legend to Fig 2 and Results). Mean ± SEM values from three separate experiments, each in duplicate dishes. *P ≤ .05, **P < .01 when comparing ATRA+ versus ATRA- control cultures. HPCs were purified by method B (see Materials and Methods).
The growth of a significant number of CFU-M colonies is FCS-mediated, in that it is absent in serum-free cultures\(^1\) (results not shown), but consistent in FCS\(^+\) cultures supplemented or not with exogenous HGFs. Furthermore, this observation is in line with our findings on adult HPC monocytic differentiation in liquid suspension culture\(^2\): thus, while adult HPCs require defined HGF combinations to develop unilineage erythroid, granulocytic, or megakaryocytic progeny in FCS\(^-\) culture condition,\(^21,25-27\) the HPC unilineage monocytic differentiation system requires not only treatment with a specific HGF combination (M-CSF + FLT3 ligand), but also FCS addition.\(^21\) The FCS requirement for monocytopoietic cell growth is seemingly mediated by serum-borne nutritional component(s) and/or HGF(s). In unpurified murine FL cells, selected HPCs similarly generate pure monocytic colonies in FCS\(^+\) clonogenic culture.\(^28,29\)

In FCS\(^+\) culture, IL-3 and GM-CSF addition induced a borderline increase of the number and a marked rise of the size of BFU-E (see Valtieri et al\(^3\)) and CFU-M colonies, while inducing formation of a few CFU-GEMM clones and a small number of CFU-GM colonies largely composed of monocytic cells and scored as CFU-M clones. Further addition of KL enhanced the formation of CFU-GEMM colonies and increased the size of all the other colony types without modifying the total number of colonies: similar results have been obtained in purified adult HPC semisolid culture\(^21,26\) (results not shown). We also describe FL primitive progenitors defined as HPP-CFCS, which display features corresponding to those of adult HPP-CFCS,\(^20,21\) except that FL HPP-CFCS generate a progeny with almost pure monocytic morphology.

The effects of retinoids on FL HPCs are dose-related and feature a drastic decrease of BFU-E/CFU-GEMM and CFU-M colonies combined with a marked rise of the CFU-G clones. ATRA\(^+\) cultures also comprised a minority of CFU-GM colonies with prevailing monocytic content (scored as CFU-M clones), while ATRA\(^-\) cultures comprised a few CFU-GM colonies largely composed of granulocytic cells (scored as CFU-G clones); thus, CFU-GM colonies showed a subtotal switch from predominantly monocytic to mainly granulocytic cell composition. We suggest that these phenomena are mediated by an erythroid/monocytic to granulocytic shift of the HPC differentiation program. Alternatively, these phenomena might be attributed to growth inhibition of BFU-E/CFU-GEMM and CFU-M colonies (comprising CFU-GM clones predominantly monocytic) combined with recruitment of CFU-G colonies (including CFU-GM clones predominantly granulocytic); however, this hypothesis contrasts with a series of observations. Thus, (1) the total number of colonies is not significantly modified by retinoid addition, (2) unicellular culture results are not compatible with the recruitment hypothesis, ie, the increase in number of CFU-G colonies in ATRA\(^+\) culture is larger than the number of nonclonogenic cells in ATRA\(^-\) culture; and finally, (3) studies on paired daughter cells show that generation of an erythroid, monocytic, granulo-monocytic, or mixed colony by a sibling in ATRA\(^-\) culture is coupled with generation of a granulocytic colony in ATRA\(^+\) culture by the other sibling (control paired daughter cell cultures performed under the same conditions without ATRA addition excluded asymmetrical divisions). Altogether, these studies conclusively dem-

### Table 1. Effect of Different ATRA Concentrations on FL HPC Colony Formation in FCS\(^+\) Cultures Supplemented With Epo Alone (100 Cells/Dish)

<table>
<thead>
<tr>
<th>ATRA Dosage (mol/L)</th>
<th>Colony No.</th>
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<th></th>
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<tbody>
<tr>
<td></td>
<td>Total</td>
<td>BFU-E</td>
<td>CFU-M</td>
</tr>
<tr>
<td>0</td>
<td>23</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>(10^{-10})</td>
<td>18</td>
<td>13</td>
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<tr>
<td>(10^{-9})</td>
<td>15</td>
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<tr>
<td>(10^{-8})</td>
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<td>3</td>
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<tr>
<td>(10^{-7})</td>
<td>1</td>
<td>0</td>
<td>1</td>
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<tr>
<td>(10^{-6})</td>
<td>0</td>
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CFU-G and CFU-GEMM colonies were absent at all ATRA concentrations.
Table 2. Effect of ATRA on FL HPC Colony Formation in FCS+ Unicellular Cultures (0.5 Cell/Well) Supplemented With Epo (3 U/mL), IL-3 (100 U), GM-CSF (10 ng), KL (100 ng)

<table>
<thead>
<tr>
<th>No. of Wells</th>
<th>No. of Cells Plated</th>
<th>No. of Colonies</th>
<th>BFU-E</th>
<th>CFU-GEMM</th>
<th>CFU-M</th>
<th>CFU-G</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATRA°</td>
<td>160</td>
<td>80</td>
<td>64</td>
<td>28</td>
<td>4</td>
<td>16°</td>
</tr>
<tr>
<td>ATRA° (10⁻⁶ mol/L)</td>
<td>160</td>
<td>80</td>
<td>64</td>
<td>28</td>
<td>4</td>
<td>16°</td>
</tr>
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* The CFU-M colony group comprised <1 of 3 CFU-GM clones containing >70% monocytic cells (see Results).
† The CFU-G colony group comprised <1 of 3 CFU-GM clones containing >70% granulocytic cells (see Results).

ATRA demonstrated a specific effect of ATRA to reprogram the differentiation program of FL HPCs to enter the granulopoietic pathway.

The ATRA induction of CFU-G colonies requires the presence of not only Epo, but also IL-3 and GM-CSF. It is possible that, upon addition of Epo alone, the HPCs reprogrammed by ATRA to generate CFU-G colonies may undergo apoptosis, due to the absence of multilineage HGFs. It is also noteworthy that the ATRA and 9-cis RA effects may be partly mediated by binding of retinoids with FCS carrier protein(s), as observed in vivo.

We previously showed that ATRA addition on purified adult HPCs causes a downmodulation of erythroid burst formation coupled with an increase of CFU-G colony number, thus suggesting, but not proving, an erythroid to granulopoietic differentiation shift. The novel results on FL shed light on this issue, in that they indicate a switch of HPCs from the erythroid or monocytic to the granulocytic-neutrophilic differentiation program.

We have also observed a striking effect of ATRA and 9-cis RA on the proliferation of early/primitive HPCs and putative HSCs (the latters assayed on the basis of the number of HPCs generated in 5-week Dexter type LTC). Thus, (1) in clonogenic HPC culture, the action of ATRA is maximal when this compound is added on freshly enriched HPCs and progressively decreases upon delayed ATRA addition: this indicates that early HPCs are the most sensitive ATRA target, while later HPCs and early hematopoietic precursors become progressively unresponsive to ATRA. (2) High concentrations of ATRA drastically reduce the number of primary HPP-CFC-derived colonies and totally abolish their recloning capacity, thus indicating a suppressive role of ATRA on HPP-CFC proliferation/differentiation and apparently self-renewal. (3) High concentrations of either ATRA or 9-cis RA drastically reduce the number of HPCs in 5-week LTC. Since in adult LTC limiting dilution experiments, the HPC number is directly related to the LTC-IC number, these results suggest an inhibitory effect of RA on the proliferation of FL HSCs.

The biological effects of retinoids are mediated through specific interaction with nuclear receptor proteins, which pertain to the steroid receptor superfamily, function as ligand-dependent regulators of gene transcription, and comprise the RAR and RXR subfamilies. ATRA is the most potent transcriptional activator of RAR, but does not efficiently bind to RXR, while 9-cis RA represents the natural ligand for RXR. The RAR/RXR heterodimer exhibits a markedly higher affinity to the response elements, as compared with RAR or RAR/RAR homodimer. Because we observed that both ATRA and 9-cis RA displayed identical

Table 3. Colonies Generated by HPC-Derived Paired Daughter Cells: Effect of ATRA Treatment on One of the Two Sibling Cells

<table>
<thead>
<tr>
<th>Control Culture°</th>
<th>ATRA-Treated Culture°</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st Paired Sibling Cell</td>
<td>2nd Paired Sibling Cell</td>
</tr>
<tr>
<td>9 Erythroid</td>
<td>7 Erythroid</td>
</tr>
<tr>
<td>2 Abortive</td>
<td>5 Granulocytic</td>
</tr>
<tr>
<td>5 Monocytic</td>
<td>1 Monocytic</td>
</tr>
<tr>
<td>4 Mixed</td>
<td>1 Granulomonocytic</td>
</tr>
<tr>
<td>18 Abortive</td>
<td>3 Erythroid</td>
</tr>
<tr>
<td>Total colony no.</td>
<td>41</td>
</tr>
</tbody>
</table>

Cumulative results from three independent experiments.
° Sibling-generated colonies (no. and type).
Our results on normal FL hematopoiesis indicate a two-level effect of RA on human embryonic/fetal HPC: (1) the inhibition of primitive HPC/HSC proliferation; (2) the shift from erythroid and monocytic differentiation program to the granulocytic one. These results strikingly mirror recent studies indicating that 5-fluorouracil–treated murine BM lymphoid-myeloid HPCs transduced with a dominant-negative RAR construct are (1) immortalized and (2) exhibit a developmental defect in the granulocytic lineage, i.e., lack of CFU-GM colony generation and an additional more advanced block in neutrophilic differentiation at the promyelocytic level, which is overcome by treatment with high concentrations of ATRA. It is of crucial interest that our results on human FL HPCs, together with studies on ATRA treatment of adult HPCs, exactly mirror those by Tsai et al.

In conclusion, we propose that retinoids play a key role in primitive HPC proliferation and are essential for HPC neutrophilic differentiation.
Fig 7. Effect of ATRA and 9-cis RA on Dexter type LTC of FL CD34+ cells (a representative experiment is shown). Nonadherent cells in LTC were counted (top) and HPCs assayed in clonogenic cultures (bottom) on a weekly basis. At 5 weeks, the LTC were terminated and the cell and HPC number in the stromal layer was evaluated.

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Dual action of retinoic acid on human embryonic/fetal hematopoiesis: blockade of primitive progenitor proliferation and shift from multipotent/erythroid/monocytic to granulocytic differentiation program

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