Effect of a New Retinoidal Benzoic Acid Derivative on Normal Human Hematopoietic Progenitor Cell Growth in Vitro

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ABSTRACT

13-cis-Retinoic acid (RA) has been demonstrated to alter hemopoiesis in vitro. We compared proliferation and differentiation effects of RA to a synthetic retinoid, (E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl]-1-propenyl|benzoic acid (TTNPB), on human normal bone marrow cells. TTNPB stimulated the in vitro growth of erythroid-granulocyte-macrophage progenitors and erythroid progenitors. Dose-response curves showed that maximal increments of erythroid-granulocyte-macrophage progenitor growth [173 ± 17% (SE)] and erythroid progenitor growth [210 ± 40%] occurred with TTNPB at 10^-8 M. With RA, there was maximal increment (173 ± 21%) for erythroid progenitors only, and that at 10^-6 M. Evaluating clonogenicity of marrow cells in the presence of the two retinoidic acid derivatives demonstrated that TTNPB and RA enhanced myeloid colony (CFU-C) growth; maximal stimulation occurred at 10^-6 M (130 ± 8% and 161 ± 5% increment for TTNPB and RA, respectively). The two retinoidic acid analogues did not alter the differentiation pattern of myeloid colonies (macrophage colonies, granulocytic colonies, or granulocyte-macrophage colonies). Replating studies showed that the formation of secondary hemopoietic colonies was not altered following incubation of hemopoietic progenitors with TTNPB or RA. These data demonstrate that TTNPB is more active than RA in stimulating the growth of hemopoietic progenitors from normal marrows. Such findings may have therapeutic implications for various hematopoietic disorders.

INTRODUCTION

Vitamin A, its metabolites, and analogues (retinoids) may participate in normal and abnormal hematopoiesis. Anemia is produced in humans who are intentionally depleted of vitamin A (2) and reduction of hematopoietic cells was noted in the bone marrow of vitamin A-deficient animals (3). Recent studies have shown that retinoids have an opposing effect on normal and malignant cell growth; i.e., they stimulate the clonal growth of normal human granulocyte-monocyte and early erythroid precursors (4, -6) while they inhibit the clonal growth of leukemic cells in vitro (7). For several years a search for active synthetic and biologically generated retinoids has been conducted in order to find retinoid molecules that have increased therapeutic potential for various cancers but have reduced systemic toxicity relative to retinoic acid. Recently several synthetic retinoids have shown great biological activity when measured by in vitro or in vivo biological assays (8, 9). One of the compounds is TTNPB. This substance was found to support the growth in the whole animal fed a vitamin A-deficient diet (10, 11); it was at least 1000 times more active than retinol in suppressing skin carcinogenesis in the mouse (11), and more than 1000 times as active as retinol or retinyl acetate in the F9 teratocarcinoma or HL-60 promyelocytic leukemia test system (8).

In the present study we have investigated the effect of TTNPB on the growth of normal human hemopoietic progenitors in vitro and have compared its activity to that of 13-cis-retinoic acid. We have found that TTNPB was superior to RA in stimulating hemopoietic progenitor growth and that the two retinoidic acid derivatives did not alter the self-renewal capacity of the hemopoietic progenitors.

MATERIALS AND METHODS

Drugs. TTNPB and 13-cis-retinoic acid were kindly provided by Hoffmann-La Roche (Basel, Switzerland). Stock solutions of TTNPB and RA were prepared in ethanol at 10^-3 M and stored at -70°C. The compounds were diluted to the required concentration in IMDM and added to cultures at the time of plating. Control plates containing only dimethyl sulfoxide did not show any effect on colony formation when diluted to the concentration of ethanol present in the experiment culture plates. TTNPB and RA were protected from bright light.

Cell Preparation. Normal human marrow cells obtained by aspiration were anticoagulated with heparin and layered on a single step Ficoll/Hypaque discontinuous gradient (1.077 g/ml) to remove mature erythro- and myeloid cells. The buoyant mononuclear cells, obtained from the interface of the gradient, were washed twice in IMDM.

Culture System for CFU-mix and BFU-E. A total of 1 x 10^6 cells/ml were cultured essentially as described previously (13) in IMDM-30% FCS-1% deionized bovine serum albumin-1 unit purified human urinary erythropoietin (1140 units/mg protein, obtained from the National Heart, Lung, and Blood Institute, NIH)-1% T-cell line conditioned medium (14) 5 x 10^-3 M 2-mercaptoethanol in a final concentration of 0.9% methylcellulose. The cells were plated in 1-ml portions in 35-mm tissue culture dishes (Lux Scientific Corporation, Thousand Oaks, CA) containing 0.1 ml of solution containing TTNPB or retinoic acid. After 14 days of incubation at 37°C in a moist atmosphere containing air-5% CO2, BFU-E and CFU-mix colonies were counted using an inverted microscope. The colonies were picked and cytospin preparations were made for Wright's Giemsa staining to assess their morphology. CFU-mix colonies were defined as colonies consisting of erythroid and leukocytic elements. Experiments were performed with 5 dishes/point.

CFU-C Culture Technique and Colony Staining. The previously described agar culture system was used (15). Briefly, 1 x 10^5 buoyant mononuclear marrow cells in 1 ml IMDM containing 15% FCS, 0.3% agar, and 0.1 ml TTNPB or retinoic acid were added to 35-mm Petri dishes containing 5% human PCM. Cultures were incubated at 37°C in a humidified atmosphere of air-5% CO2 for 10 days. Following incubation an in situ staining technique (16) was used to determine the type of myeloid colonies. Staining in sequence for α naphthyl acetate esterase (monocyte/macrophages) and naphthol AS-D chloroacetate esterase (neutrophils) permitted typing of the myeloid colonies from the same culture dish.

Cell Transfer Experiments. In these studies, individual mixed colonies were removed from primary cultures (control plates, TTNPB or RA treated cultures) on day 14 by micropipetting under sterile conditions, disrupted to obtain a single cell suspension, and transferred to a second culture medium in microtiter wells (Falcon Labware, Oxnard, CA). Secondary colony assays used the same culture conditions as primary colony assays with two modifications: 2000 cells/0.1 ml of peripheral blood mononuclear cells previously irradiated with 2000 rads were added (17); and retinoids were not included in the cultures.
The appearance of secondary hemopoietic colonies was scored after an additional 14 days of culture.

Cell Irradiation. Peripheral blood mononuclear cells suspended at 10^7 cells/ml IMDM supplemented with 10% FCS were irradiated with a 4-MeV linear accelerator at a dose rate of 200 rads/min. After irradiation, cells were immediately washed once in IMDM-10% FCS and added in appropriate aliquots to microtitre wells (17).

Statistical analyses were performed using Student's t test. Results were considered significant when P was <0.05.

RESULTS

Effect of TTNPB and 13-cis-Retinoic Acid on BFU-E and CFU-mix Growth. We tested the effect of various concentrations of TTNPB and 13-cis-retinoic acid on the growth of normal human bone marrow BFU-E and CFU-mix in methylcellulose. Dose-response curves (Fig. 1A) evaluating TTNPB at 10^{-10} - 10^{-6} M concentrations showed that maximal increments of BFU-E growth occurred with TTNPB at 10^{-9}-10^{-7} M [210 ± 40% (SE) increments over basal values]. The addition of 10^{-10}-10^{-8} M RA to culture plates enhanced the BFU-E growth maximal effect at 10^{-8} M (173 ± 21% increment) (P < 0.05) whereas 10^{-7} M and 10^{-6} M RA had an inhibitory effect.

The effect of TTNPB and RA on CFU-mix growth is shown in Fig. 1B. As can be seen, at 10^{-9} M TTNPB enhanced the growth of CFU-mix (173 ± 26%) (P < 0.05) while the slight increase in CFU-mix numbers at 10^{-10} M and 10^{-9} M was not significant, thus indicating that only TTNPB but not RA enhanced CFU-mix growth.

Effect of TTNPB and RA on CFU-C Growth. We also investigated the effect of the two retinoic acid derivatives on CFU-C growth in agar containing human PCM as a source of colony-stimulating factor (Fig. 2). The addition of 10^{-10}-10^{-6} M TTNPB and RA increased CFU-C growth; maximal increments for both substances occurred at 10^{-6} M. At this concentration, the number of CFU-C increased by 174 ± 9% and 194 ± 10% for TTNPB and RA, respectively.

Morphology of Bone Marrow Colonies in the Presence of TTNPB and RA. Using the in situ cytochemical staining technique we determined the type of myeloid colony developing in normal and TTNPB treated marrow, whether granulocytic colonies, monocyte-macrophage colonies, or granulocyte-macrophage colonies were formed. The morphology of day 10 CFU-C derived colonies is described in Table 1. In control plates the highest percentage of colonies was macrophage in type (49 ± 5%). The addition of TTNPB to the cultures caused no change in the type of myeloid colonies.

Replating Experiments. Table 2 shows the results of experiments in which cell suspensions prepared from individual mixed colonies from control, TTNPB treated, and RA treated cultures were transferred to a second culture media in a microtiter well. The results from three experiments are shown.

In these experiments 70% of each individual colony suspension was replated, the remainder being used for nucleated cell counts. Primary CFU-mix colonies ranged in size from 1.5 ×
10^3–12 × 10^3 cells per colony in control plates (mean, 5.5 × 10^3 ± 1.1 × 10^3) and 3.9 × 10^3–12 × 10^3 (mean, 8 × 10^3 ± 1.6 × 10^3) and 2.0 × 10^3–12 × 10^3 (mean, 5.8 × 10^3 ± 1.3 × 10^3) for TTNPB and RA treated cultures, respectively. Secondary hemopoietic colonies developed from primary control, TTNPB treated, or RA treated cultures had a higher frequency of CFU-C than BFU-E colonies. Overall the mean CFU-Cs per assay that were obtained per individual primary colony from control, TTNPB treated, or RA treated cultures were 3.4, 3.0, and 3.6, respectively, and those for BFU-E were 1.4, 2.4 and 1.9. One primary mixed colony from TTNPB treated cultures and two from RA treated cultures gave rise to secondary mixed colonies (Table 2).

**DISCUSSION**

Our results indicate that the retinoidal benzoic acid derivative TTNPB proved to be superior to 13-cis-retinoic acid in stimulating BFU-E growth and was equal to the latter in stimulating CFU-C growth. Furthermore, only TTNPB stimulated the growth of CFU-mix. Replicating of CFU-mix colonies revealed that a high percentage of those colony-forming cells possessed the ability to generate progenitors for secondary colonies. High self-renewal capabilities of CFU-mix and of blast colonies were also noted by other investigators (18). We also found that incubation of primary colonies with TTNPB or RA did not alter their self-renewal capability. The observation that TTNPB enhanced hemopoietic progenitor growth in culture but does not affect their self-renewal capacity might be explained by increasing the proportion of the progenitors responding to colony-stimulating factor. It might be speculated that no increase in self-renewal capacity following incubation of the progenitor cells with retinoids might be beneficial because pressure for increased self-renewal might lead to an exhaustion of the stem cells.

Recent studies on vitamin A and its derivatives have established that these compounds prevent or delay the development of various experimental tumors of the skin, lung, urinary bladder, mammary gland, and gastrointestinal tract, i.e., are cancer chemopreventing agents (12, 19). Retinoic acid also induces differentiation of various tumor cell lines (20, 21). Recently phase I clinical trials of 13-cis-retinoic acid have been conducted in patients with myelodysplastic syndromes (22, 23). It was found in these studies that some of the myelodysplastic syndrome patients treated with RA showed improvement in hematological parameters and that the toxic effects caused by RA were mild (22).

Taken together, our data indicating that TTNPB enhances normal hematopoiesis and also the findings of others as to antitumor activity by this agent suggest that, pending trials to assess its nontoxicity, TTNPB could prove a potential therapeutic agent in the treatment of preleukemic and leukemic patients.

**REFERENCES**


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