Responses of Hemopoietic Precursors to 13-cis Retinoic Acid and 1,25 Dihydroxyvitamin D₃ in the Myelodysplastic Syndromes

By Grant Swanson, Vincent Picozzi, Rodman Morgan, Frederick Hecht, and Peter Greenberg

To determine the effects of the “maturation-inducing” agents 13-cis retinoic acid and 1,25 dihydroxyvitamin D₃ on marrow cells from normal individuals and patients with myelodysplastic syndromes (MDS), we assessed marrow hemopoietic clonogenicity and differentiation response patterns to these agents. These vitamins caused increased proliferation in vitro of normal clonogenic marrow myeloid precursor cells (CFU-GM), decreased erythroid precursors (BFU-E), and no change in multipotent stem cells (CFU-GEMM). Marrow hemopoietic colony-forming cell incidence was generally subnormal in the 22 MDS patients evaluated. In vitro exposure to both agents caused various patterns of alteration of MDS hemopoietic colony and cluster formation, with similar but more pronounced effects evoked by retinoic acid. In the vast majority of MDS patients, enhanced marrow clonal granulocyte–monocyte differentiation and decreased BFU-E growth were noted after in vitro exposure to these vitamins. Correlation of biological effects was demonstrated between in vivo changes of peripheral neutrophil counts and in vitro responses of myeloid precursors for ten MDS patients treated with an eight-week therapeutic course of retinoic acid. Cytogenetic analyses indicated persisting aneuploidy or coexisting normal and aneuploid karyotypes in the cultured MDS myeloid cells and (with one exception) in native marrow cells from the treated patients. The varying responses of the MDS cells may monitor differing proportions of normal versus leukemic marrow cells susceptible to proliferative and differentiative expression on exposure to these agents.

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MATERIALS AND METHODS

Colony formation by granulocyte–macrophage (CFU-GM), erythroid (BFU-E), and pluripotent (CFU-GEMM) progenitor cells. Marrow cells were obtained by aspiration after informed consent was given in accordance with guidelines established by the Stanford Human Experimentation Committee. The cells were washed and then suspended in Iscove’s medium containing 15% fetal calf serum (FCS) (HyClone, Logan, Utah). The buoyant (<1.077 g/cm³) mononuclear marrow cells were procured after centrifugation through Hypaque-Ficoll. To determine CFU-GM, 1.5 x 10⁵ marrow cells were plated in 35-mm Lux dishes in 1 mL of a mixture of Iscove’s medium, 1.1% methylcellulose, 30% FCS, and 15% human placental conditioned medium (HPCM) as a source of colony-stimulating factor (CSF-GM), as previously described.³⁰,³¹ Myeloid colonies and clusters were generally counted after ten days of incubation at 37 °C in a humidified incubator containing air—5% CO₂. To determine BFU-E and CFU-GEMM, cells were cultured as described but with 30% FCS in the Iscove’s medium—methylcellulose mixture plus 5 x 10⁻⁵ mol/L 2-mercaptoethanol, 0.9% deionized bovine serum albumin (BSA), 1 μL/mL human urinary erythropoietin, and 1% of a T cell line conditioned medium (MoCM),²⁰ Leptalb-7 (Bovuminar—30% solution, Armour Pharmaceutical Co., Tarrytown, NY) with added sodium bicarbonate (0.08%) provided the BSA used for aiding support of BFU-E and CFU-GEMM growth. Erythroid bursts (BFU-E) and CFU-GEMM were counted on day 14 of culture. The colonies were counted using an inverted...
microscope at 30×. Representative colonies (10% to 20% of CFU-GM, myeloid clusters [possessing less than 50 cells per aggregate], BFU-E, and most CFU-GEMM) were picked, and cytospin preparations were made for Wright’s-Giemsa and alpha naphthyl acetate esterase staining to assess their morphology. All CFU-GEMM had a mixture of myeloid and erythroid cells, and some of these colonies also possessed megakaryocytes. To evaluate differentiation patterns of CFU-GM, three types of myeloid colonies were enumerated: “large cell,” compact “small cell,” and dispersed “small cell,” which were found by Wright’s-Giemsa and esterase staining to be monocyte-macrophages, blasts/cell, and dispersed “small cell,” respectively. When assessed after ten days of incubation, the distribution of HPCM-stimulated normal marrow myeloid colony types was 5% ± 1%, 18% ± 2%, and 77% ± 3% (mean ± SE), respectively.

**Drugs, stimuli.** 13-cis retinoic acid (retinoic acid), all-trans retinoic acid, retinol, and 1,25 dihydroxyvitamin D3 (vitamin D) (kindly provided by Dr Milan Uskokovic, Hoffmann-LaRoche Inc, Nutley, NJ) were diluted in absolute ethanol (final concentrations ≤0.01%) and stored at −20°C in containers gassed with nitrogen. Because these agents are light sensitive, the tubes and dishes containing them were shielded from light. CSF-GM for the intravenous studies was provided from HPCM. Partially purified human urinary erythropoietin (1,140 U/mg protein) was obtained from the National Institutes of Health. The MoCM was kindly provided by Dr David Golde, UCLA Medical Center.

**Cytogenetics.** Chromosome analyses were determined for native marrow cells and from cells incubated in vitro in clonogenic methylcellulose culture. Karyotypes from the prophase and prometaphase preparations of marrow cells thus obtained were analyzed by Giemsa banding after methotrexate/thymidine synchrony by Dr Fred Hecht and colleagues at the Southwest Biomedical Research Institute, Tempe, Ariz, as previously described. A minimum of 15 chromosome spreads were analyzed per study. To obtain karyotypes of the cells cultured in vitro, the 1-mL methylcellulose-media gels containing the cells in the tissue culture plates were solubilized after ten days in culture by adding 6 mL of Iscove’s medium plus 15% FCS (three-2-mL washes). The cell pellet obtained by centrifugation (400 g, five minutes) was resuspended, and 25 ng of Colcemid (GIBCO, Grand Island, NY) per 2 mL was added for two hours along with 15% HPCM, mixing gently every 30 minutes. The cells, obtained again by centrifugation, were resuspended in 2 mL of prewarmed 0.075 mol/L KCl for ten minutes at 37°C. Cell fixation with 4 mL 3:1 methanol/glacial acetic acid was performed by slow mixing for ten minutes. The fixed cells were centrifuged, the cell pellet recovered with 2 mL, fixed, and the cells were dispersed, incubated for ten minutes at room temperature, centrifuged, and resuspended in 1 mL of the fixative. From a height of about 15 cm, approximately 250 μL of cell suspension was added dropwise onto slides that had been presoaked in 70% ethanol and then flame dried.

**Patients.** Marrow specimens from 15 normal individuals (median age 59) and from 22 patients with MDS were studied. The 21 adult patients were elderly, median age 71 years old. The MDS patients evaluated were separated into two groups. All patients were studied at initial clinical evaluation. Group I (ten patients) were subjects who received 13-cis retinoic acid therapy orally for eight weeks after in vitro marrow culture studies, five of whom were restudied within one to two weeks after discontinuing treatment. No patient had marrow culture studies performed during retinoic acid therapy. Group II (12 patients) were subjects who had the in vitro marrow culture studies performed but did not receive retinoic acid treatment (Table 1). Diagnostic criteria for the MDS (also termed smoldering myeloid leukemia states), have previously been described and include refractory cytopenias for at least two months in patients with specifically abnormal marrow morphology. These syndromes have previously been categorized as hematopoietic dysplasia (preleukemia), smoldering or subacute myeloid leukemia (SML), and refractory anemia with excess of myeloblasts (RAEM). Hematopoietic dysplasia has been defined as showing dysmature maturation of marrow elements in at least two of the three major cell lines, including such qualitative changes as megakaryocytoid erythropoiesis, ringed sideroblasts, defective myeloid differentiation, nucleocytoplasmic asynchrony, and dwarfed or abnormal megakaryocytes. SML has been defined by these characteristics and with 10% to 30% marrow myeloblasts, some degree of myeloid maturation, and an indolent clinical course. This categorization, rather than the French-American-British (FAB) reclassification proposal, has been used to describe MDS patients for reasons of historical usage and because of ambiguity of certain FAB formulations. Eighteen of our patients had hematopoietic dysplasia and four had SML. Initial clinical features of these individuals are given in Table I. All of the patients were anemic, three were thrombocytopenic, four were neutropenic, and 11 pancytopenic. Ten study patients were treated with 13-cis retinoic acid (Accutane, kindly provided by Dr Loretta Itri, Hoffmann-LaRoche) after informed consent was given, following guidelines established by the Stanford University Human Experimentation Committee. Treated patients received 13-cis retinoic acid orally, 2.5 to 4 mg/kg/d for eight weeks, and then were reevaluated with blood counts and marrow aspirate, and assessment of marrow morphology within one to two weeks after discontinuing the drug. In six of ten treated patients, marrow cytogenetics were also reevaluated at this time. Blood counts were obtained weekly throughout the course of treatment. Repeat in vitro marrow cell culture studies were also performed in five of these patients at this time. The clinical features of these patients and in vivo results of the therapeutic trial are being reported more extensively in a separate communication. Serum retinoic acid levels achieved in individuals receiving 2.5 mg/kg 13-cis retinoic acid ranged between 0.29 and 0.91 μg/mL (1 to 3 x 10−8 mol/L) four hours after ingesting the drug (kindly performed by Harris Laboratories, Lincoln, Neb). Statistical analyses were performed using Student’s t test and chi-square test with Yates correction.

**RESULTS**

**Clonogenic proliferative responses of normal marrow hematopoietic precursors to retinoids and vitamin D.** After in vitro exposure of normal buoyant marrow mononuclear cells to 10−5 to 10−9 mol/L retinoic acid and 10−5 to 10−10 mol/L vitamin D, for the entire cell culture incubation period, maximal stimulation of CFU-GM was noted at 10−4 mol/L for retinoic acid (223% ± 15% increment over CSF-dependent basal values, P < .01) and at 10−7 mol/L vitamin D (153% ± 8% increment, P < .05) (Fig 1). In contrast, for erythropoietin-dependent BFU-E, a 30% to 50% decrement (P < .05) was caused by both agents (Fig 1). A similar decrement occurred when dimethyl sulfoxide (DMSO) rather than ethanol was used as diluent (data not shown). No change in colony formation occurred for CFU-GEMM.

To assess the specificity of the 13-cis retinoic acid effects, similar in vitro studies were performed with the related vitamin A analogues all-trans retinoic acid and retinol. Our studies with normal marrow cells showed no alteration of CFU-GM and BFU-E growth after retinol exposure in vitro at concentrations of 10−5 to 10−9 mol/L, n = 2 (Fig 2). In contrast, when using all-trans retinoic acid, moderate increments in CFU-GM growth (130% ± 12% of basal values,
Table 1. Clinical and Biological Features of Patients With Myelodysplastic Syndromes: Responses to 13-cis Retinoic Acid and 1,25 (OH)2 Vitamin D3

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Group II

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Growth patterns after in vitro exposure to retinoic acid (a through e) or 1,25(OH)2 vitamin D3 (A through E): a or A, increased CFU-GM and increased clusters; b or B, decreased CFU-GM; c or C, decreased CFU-GM and increased clusters; d or D, no change; e or E, decreased BFU-E growth. HD, hematopoietic dysplasia; SML, subacute myeloid leukemia; m, cytogenetic mosaicism.

* × 10⁶ cells per mm³.
† Per 10⁶ marrow cells.
‡ Also had in vitro marrow culture study or cytogenetics after treatment.
§ Post alkylator therapy MDS.

P < .05 occurred, with a peak at 10⁻⁴ to 10⁻⁷ mol/L, and decrements (70% ± 7% of basal values at 10⁻⁶ mol/L, P < .05) in BFU-E growth occurred, n = 3 (Fig 2).

Clonogenic proliferative and differentiative responses of MDS marrow hemopoietic precursors to retinoids and vitamin D. Twenty-two patients with MDS were evaluated with in vitro marrow culture studies. In these 22 studies, buoyant marrow mononuclear cells from 18 patients showed formation of CFU-GM, nine formed BFU-E, and two formed CFU-GEMM (Table 1, Fig 3). Colony formation, when it occurred, generally had a lower plating efficiency than that of controls, and four patients formed no colonies. No augmentation of colony formation occurred after in vitro exposure to retinoic acid (unchanged colony formation, Fig 3) or vitamin D in those patients initially lacking myeloid, erythroid, or mixed colony formation ("non-colony form-
The same patients were simultaneously evaluated for growth of the three hemopoietic progenitor cells in the absence or presence of $10^{-6}$ mol/L retinoic acid (Fig 3). For the myeloid "colony formers," several patterns of in vitro response to the vitamins were noted, with more pronounced in vitro effects being seen after retinoic acid exposure than after vitamin D exposure. Patterns of in vitro colony formation responses to in vitro exposure to 13-cis retinoic acid ($10^{-6}$ mol/L) are also shown: C, increased; D, decreased; A, no change in colony formation.

In the presence of retinoic acid, the number of CFU-GM was increased in six patients and decreased in 12 (13% to 100% change in colony formation, with the vast majority being >20% from baseline values; Fig 3). In 12 of these 18 patients, increased growth of clusters developed after in vitro exposure to retinoic acid. In vitro responses to retinoic acid of colonies and clusters together showed four growth patterns: (a) increased CFU-GM and increased clusters (six patients); (b) decreased CFU-GM, no change in clusters (five patients); (c) decreased CFU-GM and increased clusters (six patients); (d) no change (five patients, four of whom initially formed no colonies). No association was found between initial colony-forming incidence and the pattern of response to retinoic acid. Further, no correlation was noted between initial clinical features, marrow morphology, cytogenetics, or in vitro responses to retinoic acid. When grown in the presence of vitamin D, an increase in CFU-GM incidence occurred in five patients, a decrease occurred in seven, and there was no change in one, compared with control values. Decreased BFU-E growth (22% to 96%; median, 33% of control) occurred in seven of the eight tested patients having erythroid growth ($P < .05$) after retinoic acid exposure but in only three of six patients after vitamin D exposure (NS). Except for two patients whose marrows had one to two mixed colonies per $10^6$ cells plated, no CFU-GEMM were detected in marrow cells of the patients with or without vitamin exposure. Similar patterns of response of the marrow hemopoietic precursors from MDS patients occurred on in vitro exposure to retinoic acid and vitamin D (ie, concordance was found for 11 of 13 patients' myeloid growth patterns [CFU-GM, clusters], seven of eight patients' BFU-E, and all eight CFU-GEMM (Table 1, Fig 4)).

Effects on differentiation were assessed by noting whether an increase in the percentage of mature granulocyte or monocyte--macrophage type colonies occurred after exposure to these drugs. In the majority of patients, enhanced granulocyte--monocyte differentiation was noted after in vitro exposure to retinoic acid (in nine of 16 patients; 56%) or vitamin D (11 of 15 patients; 73%). Maximal changes in in vitro colony formation in MDS patients occurred at concentrations of retinoic acid ($10^{-6}$ mol/L) and vitamin D ($10^{-8}$ mol/L) that were similar to values obtained with marrow cells from normal individuals. No differences in differentiation potential were noted in cells with types (a), (b), or (c) growth patterns.

**Biological effects of the in vivo therapeutic trial with 13-cis retinoic acid.** Ten patients with MDS (group I) were treated with an eight-week course of oral retinoic acid, five of whom were restudied within one to two weeks after completion of therapy. No patients were studied during the time they were taking retinoic acid. Alterations of peripheral blood counts, marrow morphology, in vitro hemopoietic growth characteristics, and (see below) cytogenetic analyses were evaluated in these patients. After in vivo exposure to retinoic acid, these patients generally demonstrated no substantial changes from their initial colony-forming incidence or patterns of response to the drugs (Figs 3 and 5).

Correlations between in vivo hemopoietic responses to retinoic acid treatment and initial clonogenic growth patterns and in vitro responses to retinoic acid were evaluated (Table 1). Ten of the MDS patients (group I) studied with in vivo exposures to (A) 13-cis retinoic acid $10^{-6}$ M and (B) 1,25 dihydroxyvitamin $D_3$, $10^{-8}$ M and pre and post in vivo treatment with 13-cis retinoic acid.

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**Fig 3.** Marrow hemopoietic precursor [(A), CFU-GM; (B), BFU-E; (C), CFU-GEMM] colony formation in vitro from patients with myelodysplastic syndromes before and after in vivo treatment with 13-cis retinoic acid. Patterns of in vitro colony formation responses to in vitro exposure to 13-cis retinoic acid ($10^{-6}$ mol/L) are also shown: C, increased; D, decreased; A, no change in colony formation.

**Fig 4.** Proliferative responses of marrow myeloid precursors (CFU-GM) from myelodysplastic patients initially forming colonies who demonstrated increased (upper curve) or decreased (lower curve) colony formation upon in vitro exposure to (A) 13-cis retinoic acid, $log_{10} M$ or (B) 1,25 dihydroxyvitamin $D_3$, $log_{10} M$.

**Fig 5.** Proliferative responses of marrow myeloid precursors (CFU-GM incidence) from myelodysplastic patients upon in vitro exposure to (A) 13-cis retinoic acid $10^{-6}$ M and (B) 1,25 dihydroxyvitamin $D_3$, $10^{-8}$ M and pre and post in vivo treatment with 13-cis retinoic acid.
vitro marrow clonogenic assays were treated with retinoic acid. Seven of the ten patients developed increments in peripheral blood neutrophil levels during therapy (114% to 700%; median, 145%) and three had decrements (31% to 71% of basal values; median, 67%). The improved neutrophil counts occurred by six to eight weeks of therapy and returned to basal levels within two to four weeks after cessation of therapy. There were essentially no changes in the patients’ platelet counts, hemoglobin levels, or transfusion requirements during or immediately after the treatment course. No correlation was noted between in vivo responses to retinoic acid and the initial values for marrow CFU-GM, BFU-E, or CFU-GEMM incidence. All of these patients formed CFU-GM, and six had increased myeloid clusters (four patients whose neutrophils increased, two patients whose neutrophils decreased after therapy) in the absence of in vitro retinoic acid. After exposure of the marrow cells to 13-cis retinoic acid in vitro (10^{-6} mol/L), the myeloid growth patterns (a), (b), and (c) described earlier were noted. All seven patients with growth patterns (a) and (b), in response to in vitro retinoic acid exposure, subsequently developed increments in neutrophil counts after in vivo treatment, whereas the three patients with myeloid growth pattern (c) had decrements in their neutrophil counts (Table 1). These associations between in vivo responses and in vitro response patterns were statistically significantly different (P < .05). As BFU-E growth in treated patients either decreased (three patients) or (as with CFU-GEMM growth) was initially absent and did not alter (seven patients) after in vitro exposure to retinoic acid, correlations of BFU-E or CFU-GEMM responses with in vivo response were not apparent. No correlations between marrow cytogenetic findings, clinical features (eg, presence or absence of neutropenia, other cytopenias, category of MDS), or in vivo responses to retinoic acid were noted in the group I patients.

**Marrow cytogenetic analyses.** Cytogenetic characteristics and marrow morphology of native marrow cells from normal and preleukemic patients before and after in vivo exposure to retinoic acid were determined. Twenty-one of the 22 patients had marrow cytogenetics assessed. Fourteen of the 21 patients (67%) had detectable chromosome abnormalities: four elderly males had cells that lacked the y chromosome in addition to normal cells, and ten had other abnormal karyotypes. Eight patients had chromosomal mosaicism with normal and abnormal cell lines, six had only abnormal chromosomes, and six had complex cytogenetic changes. In the group of ten patients receiving retinoic acid treatment (group I), one patient had a normal marrow karyotype, three were mosaics with loss of y chromosome, and the remainder had aneuploidy (Table 1). Shared chromosome abnormalities included partial deletion of chromosome 5 (patients 10 and 21), an isochromosome 17 (patients 6 and 9), and loss of the y chromosome in cells from four males (patients 2 through 4, 17). Six patients (1 through 5, 9) had posttreatment cytogenetics assessed, and with one exception (patient 5), no change was noted in these marrow morphological or chromosomal features after therapy. Patient 5, whose marrow chromosome pattern normalized after treatment, had morphological improvement in marrow granulocyte matura-

tion and developed the most dramatic increase in blood neutrophil counts. In this patient, all of these parameters reverted to baseline values within one month after discontinuing treatment. No correlation was evident between marrow cytogenetics and the patients' clinical features, response to therapy, or in vitro hemopoietic growth patterns (Table 1).

Cytogenetic characteristics of both native marrow cells and marrow cells cultured in vitro from normal and MDS patients were determined. Cells stimulated by HPCM in methylcellulose clonogenic cultures were examined in the presence or absence of 10^{-4} mol/L retinoic acid exposure. Bone marrow cells cultured in vitro from two normal individuals (possessing normal marrow cell karyotypes) were analyzed and yielded normal karyotypes with or without in vitro retinoic acid exposure. Marrow cells from three MDS patients were examined in this fashion. One patient (12) had normal native marrow cell chromosomes and type (a) in vitro myeloid growth pattern responses to retinoic acid. This patient had normal metaphases demonstrated in cells obtained from methylcellulose gel cultures in the presence or absence of retinoic acid. Two MDS patients with either aneuploidy (patient 1) or a mixture of normal and aneuploid karyotypes (patient 15) were also evaluated. Patient 1's cells yielded chromosomes with the same abnormal karyotype as the native marrow in both the presence (wherein a 20-fold increase of clusters was generated) and the absence of in vitro exposure to retinoic acid. Both the normal and the abnormal karyotypes initially present in marrow cells from patient 15 were found after retinoic acid exposure in vitro, but only normal karyotypes were demonstrable in the absence of retinoic acid. Patient 1 also had persistence of his initial native marrow cytogenetics after in vivo treatment with retinoic acid.

**DISCUSSION**

These data describe clonogenic hemopoietic precursor proliferative and differentiative responses upon in vitro exposure of marrow cells from normal individuals and 22 patients with MDS to 13-cis retinoic acid and 1, 25 dihydroxyvitamin D_3. Confirming previous reports,13,14 we showed augmentation of normal CFU-GM colony formation after in vitro exposure to 13-cis retinoic acid, all-trans retinoic acid, and vitamin D. The increases in normal marrow CFU-GM from vitamin D exposure were similar to those from retinoic acid but were less striking. These effects occurred at drug concentrations achievable by pharmacologic doses of retinoic acid administered in vivo (10^{-4} mol/L) but required doses higher than those attained (2 \times 10^{-10} mol/L) by treatment with 1,25 dihydroxyvitamin D_3. Substantial decrements in erythroid colony formation occurred after exposure to both drugs (again, requiring excessively high concentrations of vitamin D). The inhibitory effects on BFU-E growth were not attributable to the ethanol diluent, as control values with in vitro exposure to ethanol alone showed no such decrement, whereas use of another diluent (DMSO) in the presence of the vitamins demonstrated the inhibition. Prior studies have also shown no decrement in erythroid colony formation at the ethanol concentrations that we used (\leq 0.01%).14 As specific-
erythroid colony formation in our subjects, whereas the acid exposure in the MDS patients who had erythroid growth-promoting functions,\textsuperscript{33,35} caused no change in myeloid or erythroid colony formation in the presence of retinoic acid.\textsuperscript{33} We believe that the difference is at least partially attributable to the fact that the previous study examined circulating peripheral blood BFU-E, whereas we evaluated bone marrow erythroid precursors. Marrow and peripheral blood BFU-E have differing sensitivities to erythropoietin levels.\textsuperscript{36} Concentration-dependent effects of marrow adherent cells on in vitro erythropoiesis have been demonstrated, with inhibitory effects on erythroid precursors occurring in the presence of high concentrations of these adherent monocytic cells or their culture supernatants.\textsuperscript{37,38} Modulation of several functions of adherent cells (eg, macrophages) and subsets of T lymphocytes occurs after their exposure to retinoic acid.\textsuperscript{39} Thus, functionally disparate accessory cell populations activated by retinoic acid or the BFU-E themselves, which are present within peripheral blood \(\nu\) bone marrow, may play a role in the contrasting results of our study and that previously reported. Further investigation of this issue is needed.

Low values for marrow CFU-GM and BFU-E frequency were found in the majority of marrow specimens from the MDS patients, as previously described.\textsuperscript{41-47} The marrow cells from 18 of 22 MDS patients formed CFU-GM, nine formed BFU-E, but only two formed CFU-GEMM. The data, demonstrating marked diminution in CFU-GEMM colony formation in the MDS patients, indicate that these three precursors share the quantitative hematopoietic defects present in the disorder. The absence, rather than low numbers, of CFU-GEMM may reflect their relatively low incidence in marrow (and the limited quantity of cells plated). The addition of retinoic acid or vitamin D caused no augmentation of colony formation in those individuals initially lacking in vitro clonogenicity. The data indicate that the vitamins do not overcome the basic lesion(s) in the peripheral blood in vivo. Decreased proliferative potential of the nonclonogenic MDS hematopoietic precursors.

Increased differentiation of myeloid colonies to more mature granulocytes and monocytes was noted in the majority of MDS patients after in vitro exposure to retinoic acid (56%) or vitamin D (73% of patients). Other recent studies have also demonstrated enhanced monocyte–macrophage differentiation of colonies from normal and leukemic marrow after in vitro exposure to 1,25 dihydroxyvitamin D\textsubscript{3}.\textsuperscript{18,46} In the "colony formers," various patterns of response to retinoic acid and vitamin D in vitro were noted, with similar but more pronounced effects being seen with retinoic acid. Several patterns of myeloid growth after in vitro exposure to these agents were discerned: (a) increased CFU-GM and increased cluster formation, (b) decreased CFU-GM, (c) decreased CFU-GM and increased cluster formation, (d) no change. The varying responses of MDS cells may reflect our monitoring the growth of different proportions of normal \(\nu\) leukemic cells that were susceptible to proliferative or differentiative expression on exposure to these drugs. In addition, the presence of qualitative or quantitative differences in the accessory cells affected by these vitamins could have contributed to the diminished erythroid clonal growth patterns.

Biological effects of the relatively short (eight-week) trial of retinoic acid therapy on marrow cells were assessed in these MDS patients. After in vivo treatment of patients with retinoic acid, no clear alteration occurred in the in vitro colony-forming incidence or response to these agents for marrow CFU-GM, BFU-E, or CFU-GEMM. This finding was associated with the lack of alteration in marrow morphology or cytogenetics in virtually all of these patients. Correlations between in vivo responses to retinoic acid treatment and the responses of clonogenic myeloid cells to retinoic acid exposure in vitro were found: the seven MDS patients with patterns (a) and (b) after retinoic acid exposure in vitro subsequently developed increments in peripheral neutrophil counts after treatment, whereas neutrophil decrements developed in the three patients who had generated pattern (c) in vitro. No correlations were found between in vivo responses and values of initial marrow CFU-GM, BFU-E, or CFU-GEMM incidence in vitro or marrow cytogenetics. In a separate paper, we present more extensive data detailing the clinical features and in vivo responses of MDS patients to retinoic acid therapy.\textsuperscript{33}

Nonrandom marrow cytogenetic abnormalities have been reported in a substantial proportion of patients with MDS\textsuperscript{25,52} and were present initially in 14 of our 21 patients (67%) so evaluated. The recurring chromosomal abnormalities we found were partial deletion of the chromosome 5 long (q) arm, isochromosome 17, and loss of the y. These abnormalities are associated with MDS, and isochromosome 17 may be found in the blastic phase of chronic myelocytic leukemia.\textsuperscript{12,49-52} Loss of the y chromosome is a common feature in elderly males and appears to be a reflection of aging.\textsuperscript{33} These cytogenetic findings support the concept of the MDS as representing a clinically relatively indolent spectrum of clonal hemopathies. The clonal nature of these disorders and the qualitative cellular and biological abnormalities similar to those occurring in AML strongly suggest that the patients initially demonstrate critical neoplastic features. This group of disorders appears to represent coexisting normal and leukemic clones within marrow in which the host copes effectively with the leukemic clone or is populated by a leukemic clone with potential for greater in vivo differentiation.

To more clearly determine whether normal or abnormal clonal growth was altered by retinoic acid exposure, cytogenetic analyses were performed before and after retinoic acid therapy in six patients. Five of these individuals showed no alteration in their native marrow cell karyotypes. Three of these treated patients also had karyotypic analysis performed on cells present in their myeloid colonies grown in vitro in the presence and absence of retinoic acid. These in vitro and
vivo results demonstrated, with the one exception, the persistence or enhanced generation of the clones initially present in the patients' native marrow cells on retinoic acid exposure. The data are consistent with the hypothesis that retinoic acid increased the differentiation or proliferation of the endogenous marrow myeloid clones (normal and abnormal) in the MDS patients. The implications of enhanced abnormal (possibly leukemic/preleukemic) clonal proliferation in vivo in several of our patients need to be addressed. Clonal proliferation in the bioassay system that we used is accompanied by cell differentiation. Induced differentiation of leukemic cells has been shown to be associated with their decreased capacity for self-replication and leukemogenicity. 

The data are consistent with the hypothesis that retinoic acid induced clonal proliferation in vitro (possibly leukemic/preleukemic) marrow myeloid clones (normal and abnormal) in the endogenous marrow. None of our treated patients had an increase in peripheral blood or marrow blast cells or underwent evolution to overt leukemia during the therapeutic trial. These findings provide no evidence for adverse hematologic effects of the retinoic acid exposure. However, further followup is needed to determine the long-term effects of this therapy on the patients' clinical outcomes. The temporary normalization of marrow cytogenetics in the one patient having the most striking improvement in neutrophils after in vivo retinoic acid therapy suggests treatment-induced improved growth potential of a karyotypically normal residual hemopoietic clone in this patient's marrow. The absence of correlation between marrow cytogenetic abnormalities and the in vivo or in vitro myeloid responses to retinoic acid suggest that factors distinct from detectable chromosome constitution regulate such an interplay (eg, retinoid binding proteins, effects of retinoids on accessory cells or on growth factors modulating hemopoietic precursors, alterations of cellular onc genes regulating cellular differentiation and proliferation). Such interactions between retinoids and growth factors have been demonstrated in other cell systems. 

Retinoic acid-induced differentiation is associated with altered expression of cellular oncogenes. Further evaluation is necessary and appears warranted to determine whether the natural history of MDS will be altered by more prolonged treatment of these patients with retinoic acid.

ACKNOWLEDGMENT

We are indebted to Drs. A. Barkin, T. Cohen, D.P. Cooney, J. Krakower, K. Porzig, M. Rubinstein, B. Siciq, and R. Stebbins for referring several of the patients evaluated in this study.

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Responses of hemopoietic precursors to 13-cis retinoic acid and 1,25 dihydroxyvitamin D3 in the myelodysplastic syndromes

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