Long-Term Exposure of HL60 Cells to 1,25-Dihydroxyvitamin D_3 Reduces Their Tumorigenicity: A Model for Cancer Chemoprevention (44150)

XUENING WANG, NICHOLAS M. PONZIO, AND GEORGE P. STUDZINSKI[1]

Department of Pathology and Laboratory Medicine, UMD–New Jersey Medical School, Newark, New Jersey 07103

Abstract. **Several lines of evidence suggest that 1,25-dihydroxyvitamin D_3 ($1,25D_3$) may be important in chemoprevention of human cancer. Here, we show that human promyelocytic leukemia cells HL60 cultured in the presence of 30 n*M* $1,25D_3$ (30A cells) for 3 years exhibited a reduced rate of tumor growth when injected into *nu/nu* mice, while cells grown in 40 n*M* $1,25D_3$ (40AF cells) failed to form detectable tumors in 11 out of the 12 inoculated mice. Interestingly, both 30A and 40AF cells grew approximately twice as fast as the parental HL60-G cells under tissue culture conditions, even in the presence of $1,25D_3$, to which they developed resistance. Tests of the susceptibility of these cells to natural killer (NK) cell cytotoxicity showed that 40AF, but not HL60-G or 30A cells, were targets for the murine spleen NK cells. However, lysis of 30A cells was also detected when human NK cells were used in this assay, though the effector-to–target cell ratio necessary to obtain significant lysis above background levels was higher for 30A (80:1) than for 40AF (10:1) cells. These results suggest a mechanism for the reported chemopreventive effects of sunlight-generated $1,25D_3$ or dietary vitamin D_3.** [P.S.E.B.M. 1997, Vol 215]

Evidence is accumulating that the physiologically active form of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25D_3$), and its analogs, may reduce the incidence of cancer and inhibit its progression, as recently reviewed (1–3). This evidence is provided both by epidemiological observations of the variables in the incidence of human cancer and by experimental studies using human cells or laboratory animals.

The epidemiological data link the increased incidence and the more rapid progression of such common malignancies as carcinomas of the colon, breast, or the prostate to the lack of sunlight-generated $1,25D_3$, as may occur in air-polluted cities and at high latitudes (4–6). It was pointed out that this would be especially significant in individuals with heavy skin pigmentation (e.g., black Americans), since generation of vitamin D_3 may be impaired under these circumstances (7). African-American males have the same incidence of latent, noninvasive prostate cancer as in their white counterparts, yet the incidence of aggressive, metastasizing cancer is higher in black men than in white men (8). This has suggested that serum levels of vitamin D derivatives may be reduced in men with the more aggressive forms of prostatic carcinoma, and such a correlation has indeed been reported (9).

Several studies in animals have shown that experimental carcinogenesis can be greatly retarded by the addition of a vitamin D analog to the carcinogenic regimen. For instance, recent studies in Sporn's laboratory, showed that the deltanoid Ro24-5531 markedly reduced both the *N*-nitroso-*N*-methylurea–induced mammary carcinoma in the rat (10) and the androgen-promoted carcinomas of the rat seminal vesicle and the prostate (11), while in another study dimethylbenzanthracene-induced skin tumors in mice were shown to be inhibited by $1,25D_3$ (12). The growth of human cancer

[1] To whom requests for reprints should be addressed at Department of Pathology, UMD–New Jersey Medical School, 185 South Orange Avenue, Newark, NJ 07103. This work was supported by Grants 2 RO1-CA 44722 from the National Cancer Institute and 96-37-CCR-00 from the New Jersey State Commission on Cancer Research.

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cells implanted into immunodeficient mice can also be inhibited by the administration of $1,25D_3$ or another deltanoid (13–16). It was assumed that $1,25D_3$, or a derivative, inhibits tumor growth by its direct antiproliferative action on tumor cells.

Human promyelocytic cells HL60 provide a widely studied model for the examination of differentiation-inducing and antiproliferative actions of $1,25D_3$ in suspension culture (e.g., Refs. 17–19). We have developed several sublines of HL60 cells that can grow in the presence of $1,25D_3$, and the sublines are designated by the nanomolar concentration of $1,25D_3$ in which they can proliferate (20, 21). These $1,25D_3$-resistant sublines grow faster in culture than the parental HL60 cells, and some sublines become tetraploid (20, 21). We therefore tested whether these characteristics of tumor progression are associated with increased tumorigenicity. Surprisingly, we found that long-term growth in $1,25D_3$ results in markedly reduced or absent growth of these cells when implanted as xenografts in athymic mice.

Materials and Methods

Tissue Culture. HL60-G cells (22) were grown in suspension culture at 37°C in a closed atmosphere in RPMI-1640 medium (Mediatech, Washington, DC) with 10% heat-activated, defined iron-supplemented bovine calf serum (Hyclone, Logan, UT), and with 1% L-glutamine. The HL60-G cells were passaged and fed two to three times weekly to maintain log phase growth, determined by hemocytometer counts and trypan blue (0.4%) exclusion. The $1,25D_3$-resistant cells were similarly maintained except that these sublines were split more frequently and were supplemented with nanomolar concentrations of $1,25D_3$ corresponding to the numerical designation of the subline (e.g., 30A—30 n*M* $1,25D_3$). The cells isolated from tumors growing in nude mice were obtained by carefully dissecting the tumor mass from the surrounding tissue, rinsing it once with sterile RPMI-1640 medium, mincing it under sterile conditions to obtain a single cell suspension, and culturing the cells under the same conditions as the original HL60 cells. YAC-1 (murine) and K562 (human) cell lines were used as NK-susceptible target cells for cytotoxicity assays. These cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 m*M* L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and were subcultured at regular intervals to maintain log phase cell growth prior to their use in cytotoxicity assays.

Tumorigenicity Assays. The *in vivo* growth potential of HL60 sublines was determined after injection of tumor cells into 8-week-old NCr *nu/nu* (athymic) female mice (Taconic, Germantown, NY). The mice were housed in sterilized filter-top cages, and were given sterilized food, bedding, and water. Cages were maintained in a dedicated, limited-access room within the AAALAC-accredited Research Animal Facility at the UMD–New Jersey Medical School. C57BL/6J mice were purchased from the Jackson Laboratories (Bar Harbor, ME), housed in conventional cages, and given food and water *ad libitum.* Spleen cells from these mice were used as one source of effector cells for cytotoxicity assays. For assessment of tumorigenicity, groups of six *nu/nu* mice were injected sc on the right flank with 1×10^7 or 5×10^7 cells. The incidence (i.e., tumor "takes") and tumor size were determined at regular intervals over a 60-day observation period. In mice that showed tumor growth, measurements were made using a caliper and included long (*a*) and short (*b*) diameters, as well as the thickness (*c*) of the subcutaneous tumor mass. Tumor area was calculated by multiplying *a* by *b,* and tumor volume was calculated using the formula π *abc*/6. Regardless of tumor size, mice were sacrificed if rupture of the tumor through the skin occurred. Otherwise, the mice were sacrificed when tumors reached a maximal size of 2000 mm^3. The protocols for these experiments were approved by the institution's Animal Care and Use Committee.

Flow Cytometry. The details of the procedures have been described before (23). Briefly, aliquots of 10^6 cells were washed twice with cold phosphate-buffered saline (PBS) and suspended in 70% ethanol at –20°C overnight. The cells were then washed twice with PBS and incubated with 100 U/ml of RNase (BMB, Indianapolis, IN) at 37°C for 1 hr. The cells were again washed twice with PBS and the pellet stained with a 10 μg/ml solution of propidium iodide (Sigma Chemical Co., St. Louis, MO) for 1 hr. Another aliquot of 10^6 cells was washed twice with PBS, then incubated with 0.5 μl each of the commercial preparations of MY4-RD1 and Mol-FITC antibodies (Coulter, NJ) at 25°C for 45 min to analyze the expression of surface cell markers CD14 and CD11b, using the procedures recommended by the manufacturer. The fluorescence of the cells was determined using an Epics Profile II instrument (Coulter Electronics, Hialeah, FL) as described previously (23). Cell-cycle distribution was determined using the Multicycle Software Program (Phoenix Flow Systems, San Diego, CA).

Cytotoxicity Assay. Cytotoxic activity was tested in a [^{51}Cr]-release assay as previously described (24, 25). Briefly, 100 μCi of ^{51}Cr (as sodium chromate) was added to 5×10^6 target cells in 0.5 ml of RPMI-1640 medium containing 10% FCS (Hyclone, Logan, UT), and the cells were incubated at 37°C in 5% CO_2 for 90 min. Following incubation, the cells were washed and resuspended in the medium to a concentration of 2×10^5/ml. In order to test different Effector/Target (E/T) cell ratios, effector cells were adjusted to various indicated concentrations and added to a fixed number of [^{51}Cr]-labeled target cells. One hundred microliters of effector cells and 100 μl of target cells were added to wells of Falcon U-bottomed microtest III plates (Falcon, Oxnard, CA) and incubated at 37°C in 5% CO_2 for 4 hr. Following incubation, the microplates were centrifuged at 1000 rpm for 10 min to pellet the cells, 100-μl aliquots of supernatant were removed from each well, and samples were counted in a Beckman Gamma 8000

counter. Spontaneous isotope release was obtained from supernatants of target cells incubated in medium alone, and maximum isotope release was determined from cultures in which target cells were incubated with 1% NP-40. Percentage of specific ^{51}Cr release in wells containing effectors and targets (experimental) was calculated as follows:

$$\frac{\text{cpm (experimental)} - \text{cpm (spontaneous)}}{\text{cpm (maximum)} - \text{cpm (spontaneous)}} \times 100$$

Results

Tumor Formation and Growth of Xenografts of HL60 Cells in Nude Mice. Groups of athymic (*nu/nu*) mice were injected sc with 1×10^7 or 5×10^7 cells of the various HL60 sublines, and the mice were observed for palpable tumor growth over a 2-month period. Data for the first 39 days are shown in Figure 1. Beyond this time, mice with growing tumors were sacrificed because the tumor mass reached maximal size or ruptured through the skin. Importantly, however, mice that did not develop tumors within 39 days never showed evidence of tumor growth over the entire 60-day observation period (data not shown). At a dose of 5×10^7, cell growth occurred in 100% of mice injected with 30A cells and 83% (5/6) of mice injected with the parental G line (Fig. 1A). Only one of six mice injected with 40AF cells showed growth at this cell dose. At a dose of 1×10^7 cells, none of the 40AF-injected mice showed tumor growth over the entire 60-day observation period (Fig. 1B). In marked contrast, 100% of the 30A-injected and 70% of the G-injected mice had palpable tumor nodules at this time (Fig. 1B). This exceeds the proportion of tumor "takes" reported as 38% for HL60 cells at 45th passage after original establishment in culture (26).

Tumor size also varied among the three groups of mice injected with 1×10^7 HL60 sublines (Fig. 1C). While measurable growth was not seen in 40AF-injected animals, mice injected with the parental G cells had tumors with a mean size of 1600 mm^3 at Day 39, and 30A-injected mice showed tumors with a mean size of almost 600 mm^3 over the same time period (Fig. 1C).

It was important to ensure that no metastatic tumor growth occurred in the HL60-injected mice, particularly in those mice injected with the 40AF cells. Therefore, upon sacrifice each mouse underwent a thorough necropsy examination, and tissue samples were obtained for routine histology. None of the HL60-injected nude mice, regardless of the presence or absence of a subcutaneous tumor mass, showed evidence of metastatic growth in any of their organs by gross examination. The microscopic evaluation of tissue samples was likewise unremarkable.

Characteristics of Cells Derived from Tumors When Grown in Suspension Culture. To determine whether the HL60 cells retained the same characteristics after *in vivo* passage in athymic mice, representative mice were sacrificed. The subcutaneous tumor nodules were excised and teased with fine-toothed forceps to obtain a single cell suspension. The cells were then reestablished in tissue culture, and their rates of proliferation, DNA content, phenotype, and sensitivity to $1,25D_3$ were assessed. Enumeration of cell numbers demonstrated that, just as with the nonimplanted cells (20, 21), the recovered 30A and 40AF cells grew at approximately twice the rate of growth of parental cells (data not shown), the reverse of the situation seen in the *in vivo* system (Fig. 1C).

Flow cytometric determination of the cell-cycle distribution showed DNA profiles that could not be distinguished from the cells of each subline prior to injection into mice. In particular, the near-diploid DNA content of the G and 30A sublines, and the hypotetraploid DNA content of 40AF cells (21) obtained from the only mouse with a 40AF tumor were maintained (Fig. 2A). The phenotype, however, assessed by the upregulation of the expression of the surface molecule CD14 in response to $1,25D_3$, was slightly altered by passage through the animal (Fig. 2B). After *in vivo* passage, the cells of all three sublines increased the expression of this marker of monocytic differentiation when grown in the presence of $1,25D_3$. Expression of CD11b antigen, another marker of myeloid differentiation, was similarly affected by *in vivo* passage (data not shown).

Susceptibility of HL60 Cells to NK Cytotoxicity. To determine if the various HL60 sublines could be lysed by murine NK cells, the subline cells were used as targets in a

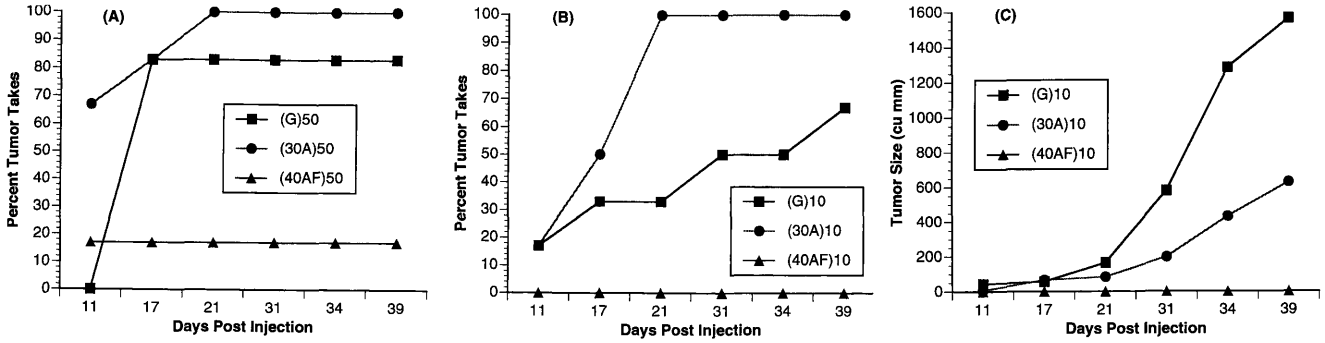

Figure 1. *In vivo* growth of HL60 sublines. Groups of athymic "nude" mice (6/group) were injected sc on the right flank with parental (G), or cells from two variant sublines: 30A, grown continuously *in vitro* in 30 n*M* $1,25D_3$; and 40AF, grown continuously in 40 n*M* $1,25D_3$. The percentage of mice that showed sc tumor growth is presented in panel A for an injection dose of 5×10^7 cells, and in Panel B for an injection dose of 1×10^7 cells. Tumor size (mm^3) was determined at regular intervals (C) as described in Materials and Methods.

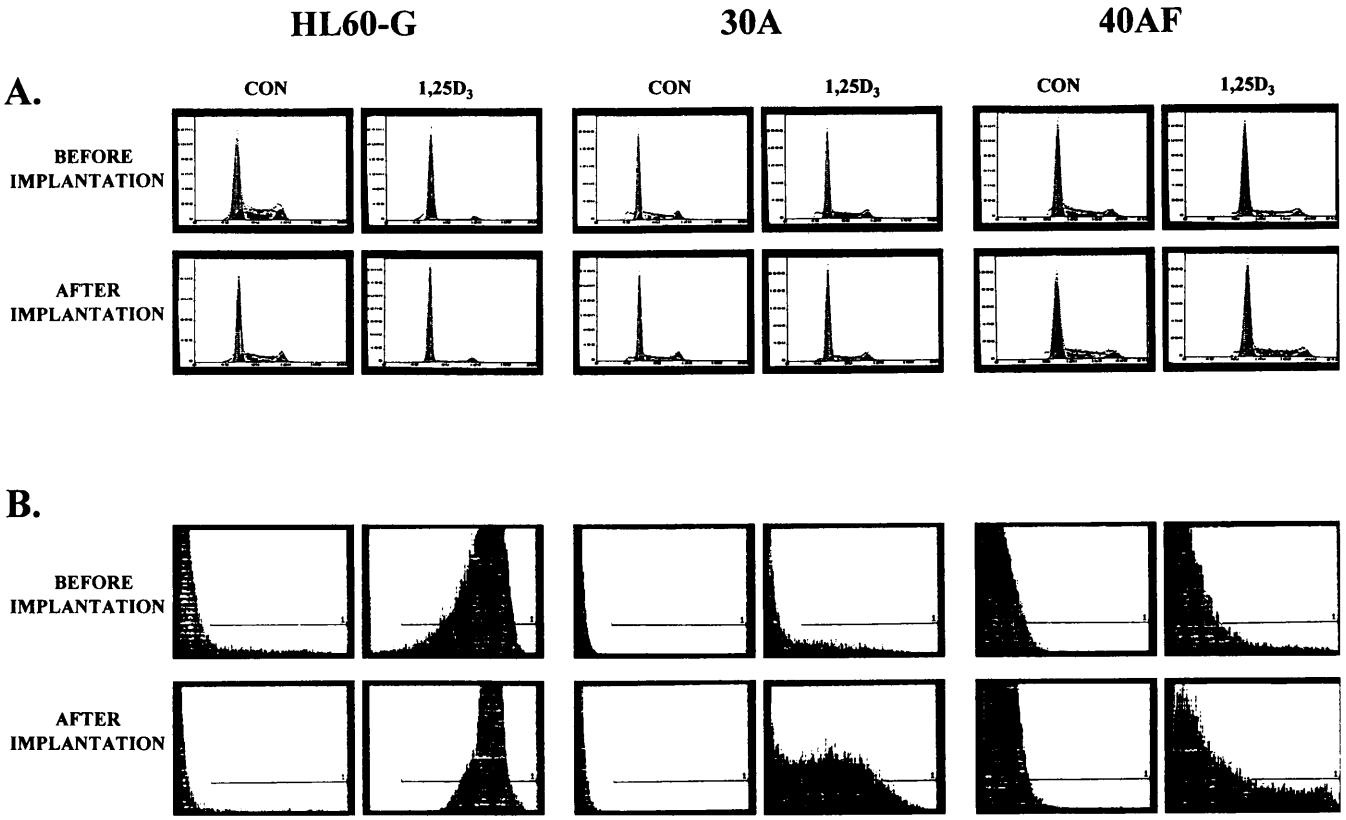

Figure 2. Comparison of the effects of $1,25D_3$ on (A) cell DNA content and cell cycle distribution and (B) cell surface expression of CD14, a marker of monocytic differentiation in HL60 sublines, before injection into nude mice, and after their reestablishment in tissue culture following recovery of the cells from the *in vivo* tumor mass. In each case, treatment with $1,25D_3$ was 10^{-7} *M* for 96 hr. The most prominent peak to the left of each panel represents the G1-phase cells, the small peak on the right represents G2-phase cells; in between there is a continuum of S-phase cells. Note that in Panel A the G1 peak of 40AF cells shows DNA content only slightly less than twice the DNA content of G1 peaks in HL60-G and 30A cells. Also, the S phase is almost absent in $1,25D_3$-treated HL60-G cells, but is evident in the $1,25D_3$-resistant 30A and 40AF, as described in detail previously (20, 21). (B) *x* axis = log of fluorescence intensity; *y* axis = relative cell number. The horizontal line in each frame indicates fluorescence that exceeds background fluorescence determined with isotype matched IgG. This experiment was repeated three times with essentially identical results.

4-hr [^{51}Cr]-release assay. The NK-susceptible murine target cell line YAC-1 was also included for comparison. As shown in Table I, YAC target cells were killed by splenic effector cells obtained from C57BL/6J or the NCr *nu/nu* mice at E/T ratios between 80 and 10. Among the three HL60 targets, only the 40AF cell line showed any lysis at all when tested against the same murine effector populations. Neither the HL60-G parental line nor the 30A subline showed lysis above background levels in this 4-hr cytotoxicity assay using murine effector cells. Indeed, even at a higher E/T ratio of 160 the HL60-G and 30A target cells showed only background levels of isotope release (data not shown).

Table I also shows the results of a similar 4-hr [^{51}Cr]-release assay in which human peripheral blood mononuclear cells were used as the effector population against HL60 subline targets. In this experiment, the human NK susceptible target cells K562 were used to document the activity of NK effector cells. The results of this assay showed an inverse correlation between the susceptibility of the various HL60 subline targets and their growth potential *in vivo*. Thus, the parental HL60-G cell tumors in nude mice were the least susceptible to human NK cells, and the 40 AF cells, which failed to grow *in vivo,* were the best NK targets in the *in vitro* cytotoxicity assay. Finally, the 30A subline, which showed moderate growth potential *in vivo* (compared with the parental G line), showed susceptibility to human NK cell activity that was intermediate between the NK-susceptible 40AF cells and the NK-resistant parental G cells. Thus, these data are consistent with the notion that NK cells present in athymic mice partially (30A cells) or completely (40AF cells) destroyed the injected leukemia cells.

Discussion

Development of $1,25D_3$ resistance is accompanied by a more rapid growth rate of the cells in suspension culture, due in part to an upregulation of the Sp1 transcription factor activity (21), so it is particularly striking that growth of these cells as xenografts in athymic mice, in the absence of $1,25D_3$ addition, is either slower than in the parental HL60 cells, or does not take place at all.

Although athymic "nude" mice are deficient in mature T cells, other cell types, including NK cells and macrophages, are fully functional and, following appropriate

Table I. Susceptibility of HL60 Sublines to Murine and Human NK Cell Cytotoxicity[a]

Effector cell (E)[b]	Target cell (T)	% Specific [^{51}Cr]-release at E/T ratio			
		10	20	40	80
C57BL/6 spleen	YAC-1	6.1	9.3	10.7	17.2
	G	−0.9	−1.0	−1.3	−0.8
	30A	−6.9	−8.0	−9.0	−5.7
	40AF	3.3	3.8	3.2	4.6
NCr "nude" spleen	YAC-1	3.4	5.0	10.2	11.2
	G	0.1	−0.2	−1.2	0.1
	30A	−7.4	−2.3	−7.9	−7.6
	40AF	3.1	3.0	3.4	2.8
Human PBMC	K562	55.0	59.3	69.1	86.2
	G	0.6	0.8	0.7	0.1
	30A	−0.3	2.7	4.5	5.2
	40AF	5.0	10.1	16.5	17.3

[a] Effector (E) cells were cultured in 96-well microplates with [^{51}Cr]-labeled target (T) cells at the indicated E/T cell ratios. After 4 hr, the plates were centrifuged to pellet cells, and an aliquot of cell-free supernatant was counted to determine isotope release.
[b] Single cell suspensions of murine effector cells were obtained from the spleens of the indicated mouse strains. Human peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Hypaque separation of whole blood.

stimulation, can exhibit cytotoxic effector function against xenogeneic target cells (27, 28). Therefore, the susceptibility of 40AF cells to murine NK cells provides a likely explanation for the lack of tumor "takes" when these cells are injected into athymic mice. The one exception in which 40AF cells formed a tumor is likely to have been due to low NK activity in that particular mouse. On the other hand, 30A cells showed no detectable lysis when they were exposed to murine NK cells, so the reason why the 30A tumors grew more slowly in nu/nu mice than the tumors formed by the parental cells is not clear. However, the 30A cells were targeted by human NK cells, so it is possible that the species barrier between human target cells and murine effector cells reduced the sensitivity of the 4-hr *in vitro* assay. It seems plausible that there was a significant ongoing attack on the 30A cells by NK cells in the mouse, reducing the number of cells in the tumor, though insufficient to prevent tumor formation. Thus, a prolonged exposure of HL60 cells to $1,25D_3$ appears to increase their sensitivity to NK cell cytotoxic activity. This suggests that growth of $1,25D_3$-treated HL60 cells in the athymic mouse results from an inhibition of the attack by NK cells.

The $1,25D_3$-resistant sublines were derived by culturing HL60 cells in stepwise increasing concentrations of $1,25D_3$ over a period of many months (20). Cells of series A, used in this study, were selected for nonadherence to flask surfaces. This accelerated the selection of cells with resistance to the differentiating effects of $1,25D_3$ on HL60 cells, since expression of several adhesion molecules is a feature of $1,25D_3$-induced monocytic differentiation (e.g., Ref. 29). Therefore, it appears that the $1,25D_3$-resistant cells acquired the ability to downregulate the expression of surface molecules required for adhesion, and this downregulation may have included the antigens which provide the inhibitory signals for the NK cell cytotoxic attack. The nature of these antigens remains to be elucidated, but it is also possible that other surface changes contribute to the reduced tumorigenicity of $1,25D_3$-resistant HL60 cells. For instance, it was recently reported that the downregulation of the expression of the type 1 insulin-like growth factor receptor results in the loss of the metastatic phenotype in murine carcinoma cells (30). Thus, the results reported here may be a part of a general cellular scheme to regulate cell phenotype, and the possible physiological relevance of this scenario is increased by the hormone-like nature of $1,25D_3$, and therefore its constant presence throughout the body.

Thus, these are two possible mechanisms that can explain these observations. First, $1,25D_3$-resistant HL60 sublines may express relevant cell surface "antigen(s)," lacking on the parental HL60-G cells, which make them more susceptible to immune attack by NK cells. Alternatively, the HL60-G parental cells may produce a factor that inhibits NK cell cytotoxicity, thus allowing their unrestricted growth *in vivo* and NK resistance *in vitro*. The $1,25D_3$-resistant sublines, however, fail to produce this inhibitory factor, which makes them susceptible targets for cytotoxic effector cells. These two possibilities are not mutually exclusive, and more extensive experiments are underway to investigate these and other mechanisms that may reduce tumorigenicity of cells adapted to a wider range of $1,25D_3$ concentrations.

The system described here allows us for the first time to distinguish the tumorigenicity-reducing effects of $1,25D_3$ exerted directly on tumor cells from those that influence the animal as a whole when $1,25D_3$ or an analog is injected into the tumor-bearing animal. We show that a prolonged exposure of HL60 cells to elevated levels of $1,25D_3$ alters the cell surface so that the cells become targets for the cytotoxic activity of murine and human NK cells. This can be one mechanism through which $1,25D_3$ derived from sunlight-generated or dietary vitamin D_3 retards the progression of human cancers.

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