Effect of 4-Hydroxyphenylretinamide and Retinoic Acid on Proliferation and Cell Cycle of Cultured Human Breast Cancer Cells [1]

Christian Marth, [2] **Günther Böck,** [3] *and* **Günter Daxenbichler** [2,4]

ABSTRACT—The synthetic retinoid 4-hydroxyphenylretinamide (HPR) showed antiproliferative effect on cultured human breast cancer cells, which were sensitive to retinoic acid (RA) too. Investigation of the cell cycle by flow cytophotometry showed a significant increase of cells in the S-phase of the cell cycle after 24 hours of RA treatment, whereas HPR did not show this effect. After 4–7 days of retinoid treatment, the percentage of resting cells increased. The influence on the cell cycle and the antiproliferative effect were more pronounced for RA than for HPR treatment in both cell lines investigated (ZR-75.1 and 734 B).—JNCI 1985: 75:871–875.

Retinoids are involved in many physiologic functions and especially in the control of the differentiation and proliferation of epithelial tissues (*1–3*). They are considered epithelial protective agents, and when exogenously administered they can prevent tumor induction by carcinogens at several organ sites (*4*). Normal phenotype of cultured embryonal carcinoma cells is reestablished when under treatment with retinoids. Moreover, proliferation of several established neoplastic cell lines is inhibited in vitro by retinoids (*5–10*). Until now, however, cancer chemotherapy with the use of retinoids has not gained importance because of its lack of objective regression in most human studies performed and because of severe side effects of prolonged high-dose treatment. Contradictory hypotheses regarding the mechanism of action of retinoids have been published. Chytil and Ong (*11, 12*) proposed that retinoids act via a specific cytoplasmic binding protein that delivers its bound retinoid to the nucleus where it could modulate gene transcription (*13*). Our laboratory and other investigators have reported an association between growth inhibition by retinoic acid and specific binding sites for this compound in breast cancer cells (*14, 15*). However, several investigators found no correlation and postulated that membrane effects or alteration of glycosylation is responsible for these effects (*16–18*). In this study we could observe that only those cells containing retinoic acid-binding protein were affected by retinoids in culture. We have focused our interest mainly on the cell cycle-specific effects of retinoids in breast cancer cells, because contradictory findings on this subject have appeared in the literature.

Treatment of HeLa or Chinese hamster ovary cells with retinoids was found to cause an accumulation in the G_1 phase of the cell cycle (*19, 20*). However, we have recently described an increased [^{3}H]thymidine uptake in mammary carcinoma cells incubated with retinoic acid with concomitant inhibition of proliferation (*14*). This finding resulted from enhanced DNA synthesis and stimulation of the cells to begin mitosis followed by a block at late S, G_2, or M phase. We have tried to elucidate the nature of this phenomenon by using flow cytometry, a more sophisticated method of cell analysis. In addition to retinoic acid as a ligand we also used HPR because of its in vivo activity in preventing carcinogen-induced mammary tumor development and because of its mild toxicity (*21*). Moderate accumulation of HPR in the liver makes it an interesting drug for clinical use.

MATERIALS AND METHODS

Retinoids.—All-trans-β-retinoic acid was a gift from Hoffman-La Roche & Co. Ltd., Basle, Switzerland. HPR was generously supplied by Dr. R. Moon (Illinois Institute of Technology, Chicago). All retinoids were dissolved in dimethyl sulfoxide at a concentration of 1 m*M* and stored at 4°C in light-protected tubes. All procedures with retinoids were performed in subdued light. The final dimethyl sulfoxide concentration in cell culture medium of each experimental as well as the control group was adjusted to 0.1% (vol/vol). For some experiments ethanol solutions were used in the same manner.

Cells.—Three human breast cancer cell lines were used in this study. BT 20, originated from a ductal carcinoma (*22*), and 734 B, established from a pleural effusion (*23*), were donated by Dr. G. C. Buehring (School of Public Health, University of California, Berkeley). ZR-75.1, which originated from ascitic fluid (*24*), was a generous gift from Dr. R. J. B. King (Imperial Cancer Research Fund, London, England). 734 B and ZR-75.1 cells contain CRABP (*14, 15*), whereas for BT 20 cells no specific binding of ^{3}H-retinoic acid was observed.

Cell culture.—Breast cancer cell lines were grown in Eagle's minimum essential medium with Earle's salts

ABBREVIATIONS USED: CRABP=cytoplasmic retinoic acid-binding protein; HPR=4-hydroxyphenylretinamide; PBS=phosphate-buffered saline.

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[2] Department of Obstetrics and Gynecology, University of Innsbruck, 6020 Innsbruck, Austria.

[3] Department of General and Experimental Pathology, University of Innsbruck.

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supplemented with L-glutamine (2 m*M*) (Eurobio, Paris), 10% fetal bovine serum (Eurobio), gentamicin (50 μg/ml; Serva, Heidelberg, Federal Republic of Germany), and amphotericin B (5 μg/ml; Arcana, Austria), as recently described (*25*).

Growth effects of retinoic acid and HPR were studied at concentrations ranging from 0.1 n*M* to 1 μ*M* either by constructing growth curves or determining cell number after 14 days of retinoid treatment. Cells were cultured for that purpose in 24-well tissue culture plates (Nunc #146485, Roskilde, Denmark) and enumerated by an electronic particle counter (Coulter Electronics Ltd., Dunstable, England) after trypsinization, as reported (*25*). The culture medium was renewed every 3d day.

Flow cytometry.—Cells were inoculated into Costar 6-well tissue culture plates (Costar #3506; Bathoevedorp, The Netherlands) 5×10^5 cells/well) and allowed to attach overnight. To obtain a partial synchronization, we treated the cells for 3 days with 1 μ*M* 4-hydroxytamoxifen (*26*). Cells were then washed twice with complete medium; and retinoic acid, HPR, or dimethyl sulfoxide alone was added to obtain a concentration of 1 μ*M* retinoid and 0.1% dimethyl sulfoxide. Cells of six wells as the control were immediately detached and cells of four wells in each group were detached on the following days with the use of trypsin (0.05%)-EDTA (0.02%) in PBS (Seromed, Munich, Federal Republic of Germany), centrifuged (200×*g*, 10 min), and resuspended in PBS. DNA staining was performed according to Taylor (*27*). Cells were then centrifuged again and resuspended in 250 μl propidium iodide solution (50 μg/ml PBS; Sigma Chemical Co., St. Louis, MO) containing 1% Triton X-100 (Serva). The nuclear suspension was gently agitated through a 22-gauge needle and filtrated through a 50-μm mash screen to remove clumps. Ribonuclease A (type I-A; Sigma Chemical Co.) was added in some experiments directly to the stained cell preparation 5 to 15 minutes before analysis to yield a final concentration of 1 mg/ml. Doublets or triplets constituted less than 3% of the total nuclear suspension. Fluorescence of 10^4 propidium iodide-stained nuclei per well was analyzed with a FACS III (Becton and Dickinson, Sunnyvale, CA). The number of cells in $G_0 + G_1$ and in $G_2 + M$ phases of the cell cycle were obtained by automatic integration of the first and second peak, respectively. Cells in S-phase were estimated by integration of cells between the upper boundary of G_0-G_1 peak and the lower boundary of G_2-M peak. Coefficient of variation of the mean value of the G_0-G_1 peak was less than 2% ($n=6$). More than 90% of the nuclei were commonly recovered. Highest variations were observed for repeated estimations of the fraction of cells in S-phase (coefficient of variation <20%). For these cell cycle studies, cells were refed each 3d day with fresh media containing fresh supplements.

Statistical evaluation.—Most of the data were analyzed with the use of nonparametric tests as the Wilcoxon rank sum test. Multiple comparisons were performed by the Kruskal-Wallis one-way analysis of variance by ranks or by the Link and Wallace test (*28*).

RESULTS

Growth Effects of Retinoic Acid and HPR

BT 20, a CRABP-deficient and retinoic acid-resistant cell line (*14*), was not affected by retinoic acid and HPR (table 1). Proliferation of 734 B as well as ZR-75.1 cells was inhibited by retinoic acid and HPR ($P<.01$, text-fig. 1). ZR-75.1 cells were obviously more retinoid sensitive than 734 B cells. Retinoic acid exhibited higher growth inhibition than HPR ($P<.05$) for both cell lines. Effects were dose related, and concentrations of retinoic acid higher than 10 n*M* were required to show significant growth inhibition ($P<.01$, text-fig. 2). A tenfold-to-fifteenfold higher molar concentration of HPR was necessary to achieve the same growth effects.

Cell Cycle Effects

Treatment with 1 μ*M* hydroxytamoxifen led to partial synchronization of cells in G_0-G_1 phase. Fluid change and fresh complete medium evolved an increase of ZR-75.1 cells in S-phase and a decrease of cells in the G_0-G_1 phase after 24 hours of treatment (text-fig. 3). Decrease in quiescent and increase in DNA synthesizing cells were more pronounced for RA-treated cells ($P<.01$). During the first 2 days of treatment, HPR led to a similar distribution of treated cells in the different phases of the cell cycle compared to that of the control group (difference not significant). A significant rise of cells in G_2 or M phase was observed mainly on day 2, and retinoic acid amplified this effect. After 4-7 days of retinoid treatment, the fraction of cells in G_0 or G_1 phase was higher for retinoic acid- or HPR-treated cells than that in the control group ($P<.02$). The two retinoid-treated populations were statistically not distinguishable.

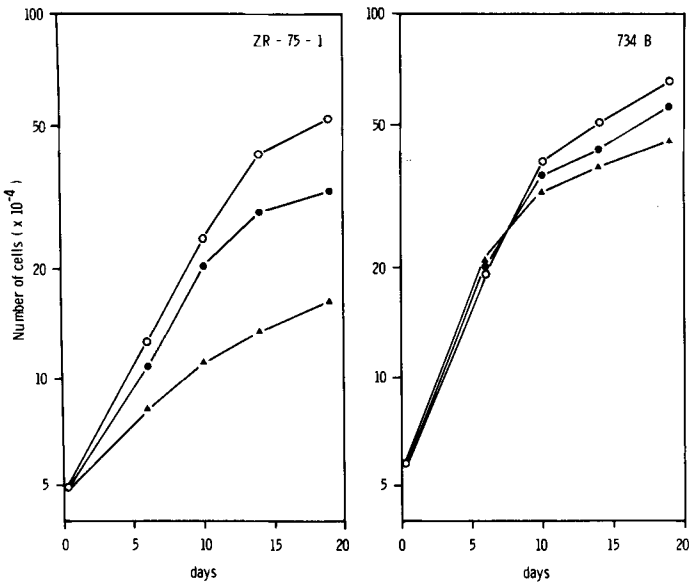
This increase of cells in the S-phase after an incubation period of 2 to 4 days with retinoic acid and a partial accumulation of cells in G_0-G_1 after longer treatment were also observed without synchronization or if ethanol instead of dimethyl sulfoxide was used as the solvent for the retinoids (data not shown).

The second retinoid-sensitive breast cancer cell line tested, 734 B, showed an increased percentage of cells in S-phase ($P<.01$) or G_2-M phase ($P<.01$) after 24 hours of retinoic acid treatment (text-fig. 4). HPR was not able to affect significantly the cell cycle of treated cells compared to that of the control group. In analogy to ZR-75.1 cells, we observed after 7 days of incubation a higher percentage of 734 B cells in G_0 or G_1 phase when they

TABLE 1.—*Growth effects of retinoic acid and HPR on BT 20 cells*

Treatment[a]	No. of cells ($\times10^{-5}$)[b]
Vehicle alone	5.26±0.42
Retinoic acid, 1 μ*M*	5.15±0.31
HPR, 1 μ*M*	5.38±0.48

[a] 6×10^4 BT 20 cells per well were incubated and grown for 14 days.
[b] Mean ± SD of 8 wells.

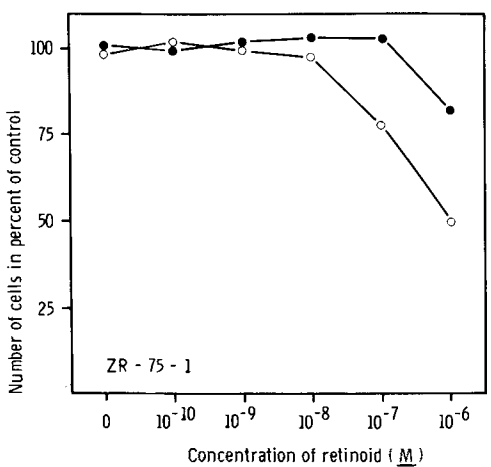


TEXT-FIGURE 1.—Effects of retinoic acid and HPR on the proliferation of ZR-75.1 (ZR-75-1) and 734 B cells. The cells were continuously treated with 0.5 μM retinoic acid (▲), 0.5 μM HPR (●), or the vehicle alone (○) as a control. Results are plotted as the means of four wells counted. Variation coefficient was always lower than 10% and, for clarity, was not shown.

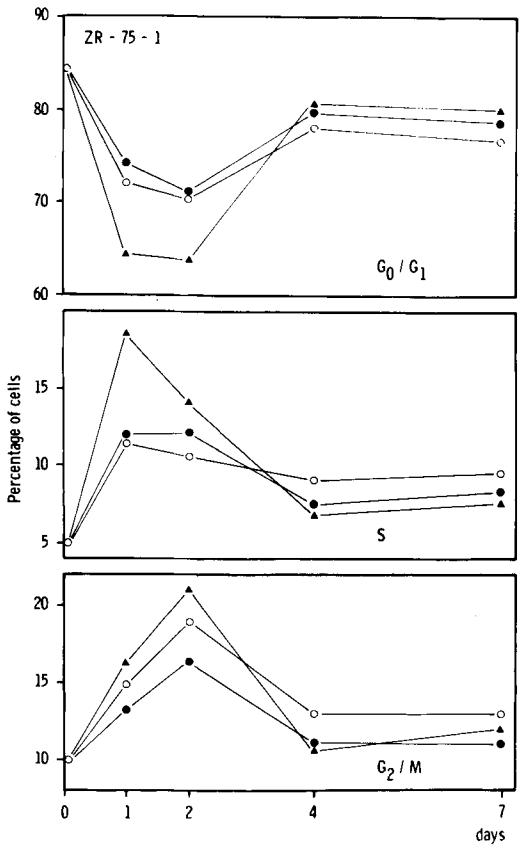
were treated with retinoic acid or HPR ($P<.01$). Retinoic acid-treated cells accumulated more in G_0-G_1 phase than did the HPR-incubated population ($P<.05$).

DISCUSSION

HPR was described by Moon et al. (*21*) as an effective agent for the inhibition of development of breast cancer induced in rats by *N*-methyl-*N*-nitrosourea. Its activity was lower than that of retinyl acetate, a highly potent

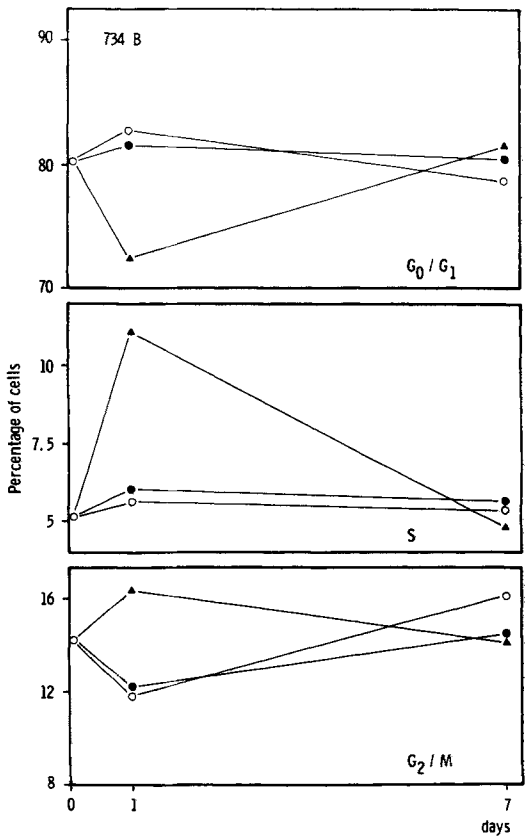


TEXT-FIGURE 2.—Dose dependence of growth effects of retinoic acid and HPR on ZR-75.1 (ZR-75-1) cells. 1.5×10^5 cells were seeded and then treated with retinoic acid (○) or HPR (●) for 10 days. *Each point* represents the mean of four wells counted, and results are expressed as percent of the cell number in the control groups (100% corresponds to 6.1 and 6.2×10^5 cells, respectively).



TEXT-FIGURE 3.—Effects of retinoic acid and HPR on the cell cycle of ZR-75.1 (ZR-75-1) cells. Cells were treated for 3 days with 1 μM 4-hydroxytamoxifen. Thereafter (day 0), the medium was changed and supplemented with 1 μM retinoic acid (▲) or 1 μM HPR (●) or with 0.1% (vol/vol) dimethyl sulfoxide alone as control (○). Flow cytometry and evaluation were performed as described in "Materials and Methods." *Top panel* shows percentage of cells in G_0 and G_1 phases, *middle panel* shows cells in S-phase, and *bottom panel* indicates percentage of cells in G_2 and M phases. *Each point* represents the mean of four wells analyzed. For clarity, standard deviations are not shown.

retinoid in vivo (*21*). We found a growth inhibitory action of HPR on established breast cancer cells cultured as monolayer, but the activity was always lower than that for retinoic acid. It is not clear whether these two results are comparable, inasmuch as our system is only able to give an answer on the proliferation of tumor cells, whereas in carcinogen-treated rodents retinoids may exhibit both anticarcinogenic as well as growth inhibitory activity (*2*). HPR is an active agent in both systems. With its mild toxicity in vivo it could be an interesting new tool in breast cancer therapy. But even high concentrations of the retinoid were unable to block proliferation of responsive cells completely; thus HPR could be considered as an additive drug only. As the action of retinoids was proposed to depend on the presence of a specific cytoplasmic binding protein, it is surprising that HPR is not able to bind specifically to such a protein and that no active metabolites could be detected in breast tissue of treated rats (*29*). Preliminary experiments revealed that extracts of HPR-treated cells



TEXT-FIGURE 4.—Effects of retinoic acid and HPR on cell cycle of 734 B-cells. For this experiment cells were not pretreated with 4-hydroxytamoxifen, and ethanol retinoid solutions were used instead of dimethyl sulfoxide. For other particulars, *see* text-fig. 3 and its legend.

and their culture medium did not show any binding affinities; thus metabolism to free retinoic acid or other derivatives with affinity to the binding protein did not occur in a detectable manner (Marth C, Böck G, Daxenbichler G: Unpublished data). Despite this observation, only CRABP-containing cells were affected by HPR or retinoic acid. We tried in this study to elucidate cell cycle effects of retinoic acid and HPR because the inhibition of proliferation of 734 B-cells observed after 10–14 days of retinoic acid treatment was anticipated by an increased thymidine uptake (*14*). These findings are confirmed by our recent results. In 734 B as well as in ZR-75.1 cells, retinoic acid provoked an increase of cells in the S-phase. This effect was seen only during the first 2–4 days, whereas thereafter cells accumulated in G_0–G_1 phase. Therefore, we conclude that retinoic acid acts in a two-step mechanism: In an early stage of treatment DNA synthesis is enhanced, whereas after 2–4 days cell stimulation relapses and growth inhibition begins, resulting in an increase of cells in the resting phase. Interestingly, Schroder et al. (*10*) recently reported a comitogenic effect of retinoic acid on murine Swiss 3T3 cells for the first 24 hours, whereas for longer exposure they described inhibition of DNA replication. For HPR, however, we did not see this characteristic early effect of retinoic acid, but later on the increase in G_0–G_1 phase nuclei was associated with growth inhibition. We do not know if this increased amount of quiescent cells is due to a higher portion of G_0 cells resulting from a reduced growth fraction or to a longer duration of G_1 phase. Both possibilities lead to a lower number of cells that begin mitosis. However, Lotan et al. (*9*) recently showed for cultured S-91 melanoma cells an accumulation in G_1 phase under retinoic acid treatment. A block in the cell cycle at the S or G_2 phase as hypothesized by us in an earlier report (*14*) could not be confirmed in this study, but difficult demonstration may ensue for more than one block of cell cycle with our method. Since specific binding of HPR was absent, since metabolites occurring in breast cancer cells could not be detected, and since, furthermore, an early retinoic acid-like effect was not observed, we cannot exclude that HPR and retinoic acid act through two different mechanisms.

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