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Retinoic Acid Induces a G_1 Cell Cycle Block in HeLa Cells (40806)[1]

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Vitamin A is known to play a role in the normal differentiation of epithelial tissues (reviewed in (1)), but through a mechanism of action which remains to be elucidated. There are many reports in the literature which document a diverse range of vitamin A-induced changes *in vitro*. Effects on desmosome and hemidesmosome formation (2), Golgi bodies (3), mucopolysaccharide synthesis (4), glycoprotein synthesis (5, 6), adhesion (7), and interferon production and action (8) have been reported. Directly relevent to this paper are several publications that have established that vitamin A and its active analogs can dramatically inhibit the proliferation of cells *in vitro*. We have previously reported that retinoic acid reduced the saturation density of L929 cells, (12) and induced anchorage dependent growth of L929, B16C3, and HeLa cells (13). Lotan *et al.* (9, 10) have extended these findings and have demonstrated that retinoic acid can have inhibitory effects on the proliferation of a broad range of cell types. More recently, Jettan *et al.* (11) reported that certain retinoids including retinoic acid reduced the saturation density of 3T3 and 3T6 cells. These reports suggest that retinoic acid and certain other retinoids may establish normal growth controls to responsive transformed cells.

In this paper we have characterized the growth inhibition by retinoic acid of suspended HeLa cells with respect to the cell cycle. Haddox and Russell have very recently reported on a similar study involving the inhibition of Chinese hamster ovary (CHO) cells by retinol (14).

Materials and methods. HeLa cells (ATCC CCL2) were obtained from Gary Stein of the University of Florida. Cells were routinely passaged as monolayers in Eagle's minimal essential medium (MEM) supplemented with 10% bovine calf serum (BCS). Spinner cultures were established by suspending trypsinized cells at 2×10^5 cells/ml in Joklik's modified spinner MEM (GIBCO, Grand Island, New York) supplemented with 10% BCS. Spinner cultures were established for at least 1 week prior to use in these experiments.

Spinner cultures were synchronized using a double thymidine block. Thymidine was prepared as a 0.2 *M* solution in water and filter sterilized. Exponentially growing cultures of HeLa cells (2×10^5 cells/ml) were brought to 2 m*M* thymidine and the block maintained for 12 hr. The cells were released from the block by centrifugation (800*g* for 10 min), and resuspension in fresh prewarmed spinner medium. The cells were again blocked, after 9 hr, by the addition of 2 m*M* thymidine and the new block was maintained for 12 hr. Upon release from this final block, the cells were split into replicate 100-ml cultures.

DNA synthesis was assayed by [^{3}H]thymidine incorporation. Aliquots (2.0 ml) from the spinner cultures were placed in 16 × 125-mm plastic Falcon tubes (No. 2025). Each tube received 200 μl of suspension medium containing [^{3}H]thymidine (10 Ci/ml, 1.9 Ci/mmole) and the tubes were incubated for 1 hr in the tube roller at 37°C. At the end of the incubation, the contents of the tubes were collected on a Millipore manifold. The tubes were rinsed three times with Gey's balanced salt solution without calcium and magnesium and the wash material was added to that already collected on the filter. The filters were washed three times with 5% TCA and three times with

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95% ethanol. The filters were dried, placed into scintillation vials, suspended in 10 ml of scintillation cocktail, and counted.

For those experiments in which [^{3}H]thymidine incorporation was assayed in cells grown in tissue culture flasks, each flask (25 cm^2 containing 5 ml medium) received 200 μl of medium containing [^{3}H]thymidine (10 μCi/ml). The flasks were incubated on a shaker platform for 1 hr at 37°C. The cells were removed by trypsinization and collected on Millipore filters as described above. All procedures involving the Millipore manifold were conducted at 4°C using precooled solutions.

Cell densities were determined using a Coulter counter, model ZBI. Viability and cell clumping were determined by trypan blue exclusion and use of a hemocytometer.

Results. Cell cycle compartments. When a spinner culture of HeLa cells was synchronized by a double thymidine block and then released, the cells progressed through the cell cycle as a synchronized population. The cell cycle under these conditions was 18 hr in length as determined by the elapsed time between two rounds of DNA synthesis. The S phase lasted about 7 hr, G_2 1–2 hr, and, consequently, G_1 was 8–9 hr in duration.

Effect of retinoic acid on HeLa spinner culture. When a nonsynchronized, exponentially growing spinner cell population was treated with retinoic acid (RA) at 10^{-5} *M* (3 μg/ml) (Fig. 1), no inhibition of proliferation occurred within the following 24 hr but an inhibition of further cell proliferation occurred during the following 24-hr period. This observation is consistant with the hypothesis that retinoic acid established a cell cycle block shortly after mitosis. Since there would be few cells located in this region of the cell cycle (between mitosis and the hypothesized early G_1 block), when retinoic acid was added, most of the cells in the population would continue to progress through the cell cycle past mitosis. Thus, most cells would be able to divide in the first 24 hr. During the second 24 hr, however, the cells would be blocked and no increase in cell numbers would occur. The increase in the cell numbers between Days 4 and 5 in Fig. 1 was not observed if fresh retinoic acid was added daily.

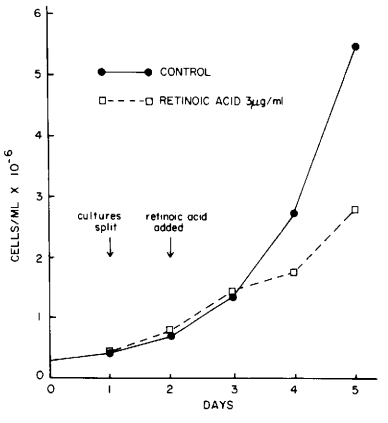

FIG. 1. Retinotic acid inhibition of HeLa cell proliferation in a nonsynchronized spinner culture. Retinoic acid (3 μg/ml), □ – – –□; control (0.1% ethanol), ●——●.

Reversal of the retinoic acid block. Since the previous experiment suggested that retinoic acid established an early G_1 block in HeLa cells, experiments were conducted to determine if release from the block would result in a synchronized progression through the cell cycle by the cell population. Spinner cultures were treated with RA at 5×10^{-5} *M* (15 μg/ml) for 24 hr during which time the cell population doubled to 5×10^5 cells/ml and at the end of which trypan blue exclusion indicated better than 95% viability. Cells were collected by centrifugation and resuspended at 5×10^5 cells/ml in fresh MEM + 10% BCS and plated at 5.0 ml per 25-cm^2 flask. Figure 2 indicates the results when the flasks were assayed for [^{3}H]thymidine incorporation into acid precipitable material at the indicated times. The cells appeared to enter a synchronous round of DNA synthesis with a peak of DNA synthesis at approximately 15 hr. This experiment demonstrates that retinoic acid induced a reversible cell cycle block in suspended HeLa cells and that release from this block by simultaneous washing out of the retinoic acid and attachment to a tissue culture flask results in a synchronous progression through the cell cycle. Since the entirety of G_1 and S phases encompass only 15–16 hr, this experiment indicates that the release from the block

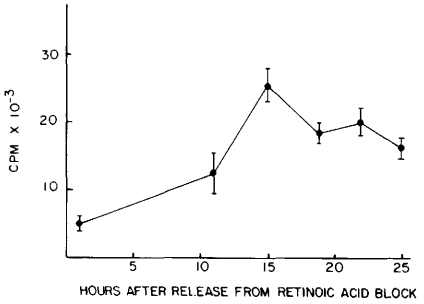

FIG. 2. Reversal of retinoic acid block by simultaneous removal of retinoic acid and attachment of cells to tissue culture flask.

does not occur immediately for the entire cell population and that the retinoic acid block occurred shortly after mitosis.

Interaction of RA with a synchronized cell population. Data in Figs. 1 and 2 indicated that cells in the presence of retinoic acid would undergo one cycle of replication and then be blocked early in G_1. To further analyze the nature of the block, we employed synchronized cultures. Preliminary experiments indicated that the addition of retinoic acid immediately after removal of the thymidine block did not inhibit DNA synthesis in that cycle but that after division, the cells would not progress through G1 and undergo DNA synthesis again. These experiments were conducted to determine how late one could delay the addition of retinoic acid after removal of the thymidine block and still inhibit the next round of DNA synthesis.

A culture of HeLa cells was synchronized by double thymidine block and at the time of release from the thymidine block was split into four replicate cultures. One culture was not treated and served as a control. The amount of DNA synthesis occurring in this control culture during the second cycle of DNA synthesis was considered to be 100% of that possible (0% inhibition). At three different times after release from the block, 3 μg/ml of retinoic acid was added to one of each of the other three cultures. The amount of DNA synthesis during the subsequent cycle in the culture receiving an early addition of retinoic acid was arbitrarily considered to be the minimum possible and was said to equal 100% inhibition. The other two flasks received retinoic acid at later periods and the amount of inhibition observed. Figure 3 shows the results from such an experiment. It can be seen that when retinoic acid was added at 3 hr after release, considerable but not complete inhibition occurred. It retinoic acid was added 9 or 12 hr after release, only minimal or no inhibition occurred. Thus, 9 hr was too long to wait and the experiment was repeated using points of addition which were earlier than 9 hr. Figure 4 shows a composite of the results of two experiments. We have concluded that there exists a point between 3 and 7 hr after the cells have entered S phase (and are still in S phase) at which retinoic acid can express 50% of its maximal inhibition. These experiments also demonstrated that the retinoic acid-treated HeLa cells were subsequently blocked at some point in the G_1 phase of the cell cycle since all of the spinner cultures doubled in cell density regardless of whether the retinoic acid was or was not added (Table 1). However, those cultures which received retinoic acid at or soon after their release from the thymidine block did not enter the second S phase.

Discussion. We have previously reported that retinoic acid would restore density dependent inhibition of growth to L929 cells (12) and anchorage dependent growth to selected transformed mammalian cells (13). These observations suggested that the acid form of vitamin A could reverse two of the most widely observed markers of transformation to the normal phenotype.

In an effort to better characterize the retinoic acid-induced inhibition of anchorage independent growth, experiments were conducted using HeLa cell spinner cultures. This technique has allowed us to investigate the retinoic acid-induced block with respect to the cell cycle.

When a population of HeLa cells was blocked by the appropriate thymidine treatment and then released from that block, the population showed a time dependent pattern of response to retinoic acid when assayed for cell number or [^{3}H]thymidine incorporation. Most important was that the population doubled within 16 hr regardless of the time of retinoic acid

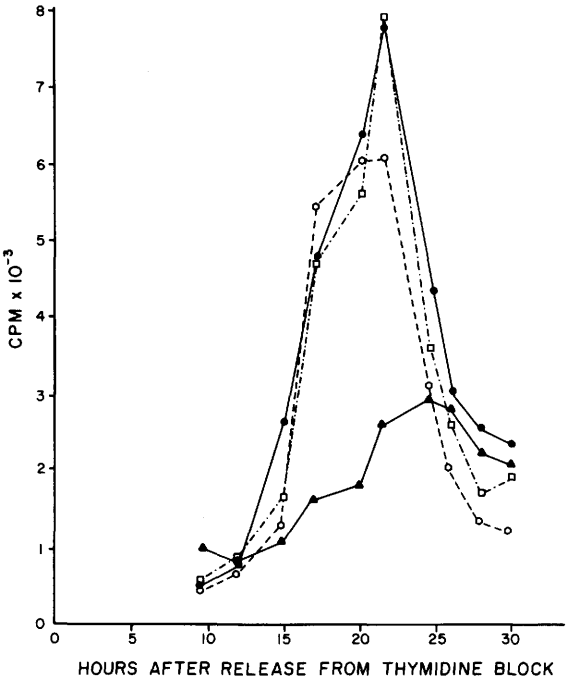

FIG. 3. Retinoic acid inhibition of DNA synthesis in the second cycle in synchronized spinner cultures following release from the thymidine block. Retinoic acid added at: 3 hr, ▲——▲; 9 hr, ○– – –○; 12 hr, □ – · – · –□; control, ●——●.

addition indicating that either retinoic acid required longer than 16 hr to act or that there was a specific blockade point occuring after mitosis. It was only after the cells passed through mitosis that any effect of retinoic acid was observed. From Figs. 3 and 4 it appears that retinoic acid applied within the first 3 hr after release from the thymidine block significantly reduced the ability of the cells to enter another S phase.

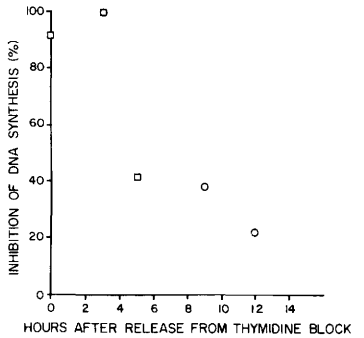

FIG. 4. Retinoic acid inhibition of synchronized HeLa cell spinner cultures as a function of the time of retinoic acid addition.

Furthermore, from Fig. 3 it can be seen that when retinoic acid is added at 3 hr, the cells which do undergo DNA synthesis are significantly retarded in their progress through G_1 since DNA synthesis is delayed. Thus, when retinoic acid was added before or during early S phase, the cells appeared to be blocked after mitosis and before S phase, indicating that some early S phase event was responsible for the subsequent block in the G_1 phase of the cell cycle. The block requires the continued presence of retinoic acid since the cells enter the S phase when the vitamin is removed (13).

Our study supports that of Haddox and Russell (14) in so far that two significantly different cell systems, employing two different vitamin A analogs, appear to respond with a cell cycle specific block in G_1. The two systems appear to differ in that retinoic acid in HeLa cells required several hours (at least 7) to block progression through the current cell cycle whereas retinol was much faster acting in the CHO cells. Even more interesting perhaps is that retinoic acid was

TABLE I. EFFECTS OF RETINOIC ACID ON CELL PROLIFERATION AND DNA SYNTHESIS IN A SYNCHRONIZED POPULATION OF HeLa CELLS[a]

Time of RA addition (hr)	Cell density and time of cell counting ($\times 10^4$/ml)			DNA synthesis (% of control)
	0 hr	16 hr	30 hr	
3	13 ± 2	21 ± 2	20 ± 3	51
9	12 ± 2	22 ± 4	35 ± 5	81
12	13 ± 2	25 ± 4	36 ± 5	89
Ethanol (0.1%)[b]	13 ± 2	20 ± 6	38 ± 4	—

[a] Data are from same experiment as presented in Fig. 3.
[b] Ethanol was the solvent for 1% vitamin.

not active in CHO cells (14). We did not study the effect of retinol on HeLa cells, having concluded earlier (13) that retinoic acid was the most active of the natural vitamin A analogs *in vitro*.

Since the normal resting state of eucaryotic cells is usually in a portion of the G_1 phase, these observations that vitamin A analogs can establish reversible G_1 cell cycle blocks in selected cell types strengthen the possibility that vitamin A may play a role in maintaining normal growth controls although perhaps under very specific conditions.

Summary. Evidence is presented that retinoic acid-induced anchorage dependent growth of HeLa cells is cell cycle dependent. Spinner cultures of retinoic acid-inhibited cells were blocked in the G_1 phase and this block was reversed by simultaneous removal of retinoic acid and attachment of the cells to a plastic tissue culture surface. Studies with a synchronized cell population demonstrated that the retinoic acid had to be added during or before late S phase if the cells were to be blocked in the subsequent G_1 phase. These findings are consistent with the hypothesis that vitamin A establishes normal growth controls to some transformed cells.

1. De Luca, L. M., *in* "Handbook of Lipid Research" (H. P. De Luca, ed.), p. 1. Plenum, New York, 1977.
2. Christophers, E., and Wolff, H. H., Nature (London) **256**, 209 (1975).
3. Yuspa, S. H., and Harris, C. C., Exp. Cell Res. **86**, 95 (1974).
4. Solursh, M., and Meier, S., Calcif. Tissue Res. **13**, 131 (1973).
5. De Luca, L. M., Vitam. Horm. **35**, 1 (1977).
6. Adamo, S., De Luca, L. M., Silverman-Jones, C., and Yuspa, S. H., J. Biol. Chem. **254**, 3279 (1979).
7. Adamo, S., De Luca, L. M., Akalovsky, I., and Bhat, P. J., Nat. Cancer Inst. **62**, 1473 (1979).
8. Blalock, J. E., and Gifford, G. E. Proc. Nat. Acad. Sci. USA **74**, 5382 (1977).
9. Lotan, R., and Nicolson, G. L., J. Nat. Cancer Inst. **59**, 1717 (1977).
10. Lotan, R., Giotta, G., Nork, E., and Nicolson, G. L., J. Nat. Cancer Inst. **60**, 1035 (1978).
11. Jetten, A. M., Jetten, M. E. R., Shapiro, S. S., and Poon, J. P., Exp. Cell Res. **119**, 289 (1979).
12. Dion, L. D., Blalock, J. E., and Gifford, G. E. J. Nat. Cancer Inst. **58**, 795 (1977).
13. Dion, L. D., Blalock, J. E., and Gifford, G. E. Exp. Cell Res. **117**, 15 (1978).
14. Haddox, M. K., and Russell, D. H., Cancer Res. **39**, 2476 (1979).

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