Vol. 102, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 16, 1981 Pages 315-321

RAPID INDUCTION OF ALKALINE PHOSPHATASE ACTIVITY BY RETINOIC ACID

David H. Reese*, Gilbert J. Fiorentino**, Alice J. Claflin **, Theodore I. Malinin **, Victor A. Politano*
Departments of Urology* and Surgery**, University of Miami School of Medicine, P.O. Box 016217, Miami, Florida 33101

Received August 3,1981

SUMMARY: Retinoic acid (vitamin A acid) increased alkaline phosphatase activity in cultured cells derived from both normal rat prostate and the Dunning R-3327 transplantable prostatic adenocarcinoma. Retinoic acid was found to be 3-4-fold more effective as an inducer of enzyme activity than retinol or retinal. In one rapidly-growing cell line (UMS-1541Q) which has a barely-detectable level of enzyme activity in the uninduced state, increased activity could be detected as early as 3-4 hours after the addition of 10 μM retinoic acid. This increase was totally blocked by actinomycin D and cycloheximide. The demonstrated rapid inducibility of alkaline phosphatase activity provides a specific marker for the action of retinoic acid at the molecular level.

Alkaline phosphatase (EC. 3.1.3.1) is one of a group of marker enzymes which has been shown to fluctuate quantitatively according to the state of tumor differentiation in sublines of the Dunning R-3327 transplantable prostatic adenocarcinoma. Compared to the original R-3327 tumor, sublines which exhibit decreasing degrees of androgen sensitivity, loss of histological differentiation, and increased growth rates are also found to exhibit correspondingly lower levels of alkaline phosphatase activity (1). An understanding of the mechanism involved in the regulation of this marker enzyme in R-3327 tumor cells would be helpful in understanding the progressive loss of prostatic tumor differentiation and the concomitant increase in tumor virulence. Recently, during the course of preliminary studies on the glucocorticoid suppression of growth in cultured cells of the G subline (2) of the R-3327 tumor, we investigated whether alkaline phosphatase activity could be induced by the synthetic glucocorticoid hormone, dexamethasone. Although alkaline phosphatase has been shown to be inducible by glucocorticoids in a variety of different cell types (3), we found no evidence that dexamethasone increased enzyme activity in R-3327 cells.

0006-291X/81/170315-07$01.00/0

In addition to glucocorticoids, numerous other agents have been implicated as regulators of various tissue-specific alkaline phosphatases (3). Riley and Spearman (4) showed that prolonged topical application of retinyl acetate to the tail epidermis of mice caused increased incorporation of radiolabeled leucine into alkaline phosphatase-positive bands fractionated by starch gel electrophoresis. This observation suggests that vitamin A and its metabolites may regulate alkaline phosphatase in some cell types. We have investigated this possibility in cultured prostatic cells of normal and malignant origin and found that retinoic acid causes the rapid, RNA and protein synthesis-dependent induction of alkaline phosphatase activity.

MATERIALS AND METHODS All cells were grown in CMRL 1415 medium (5) containing 2X sodium bicarbonate and supplemented with 10% newborn calf serum. Stock solutions of all-trans-retinoic acid, all-trans-retinol, and all-trans-retinal (Sigma Chemical Co.) were prepared in either absolute ethanol redistilled from NaOH or in dimethyl sulfoxide. The final concentration of both vehicles in all media was 0.1%.

For the determination of alkaline phosphatase activity, the monolayers were washed with phosphate-buffered saline and the cells lysed at room temperature in 0.2% Nonidet P40 (Particle Data Lab.) containing 1 mM $MgCl_2$. The cell lysates were frozen, thawed, and vigorously vortexed for 1 min prior to alkaline phosphatase and protein determinations. Alkaline phosphatase activity was measured by incubating samples of cell lysates at 37° in 0.64 M 2-amino-2-methyl-1-propanol buffer (pH 10.3) containing 16 mM p-nitrophenylphosphate and 1 mM $MgCl_2$. The reaction was stopped by adding an equal volume of 0.2 N NaOH and the amount of p-nitrophenol liberated was measured spectrophotometrically at 410 nm. Protein was determined by the method of Bradford (6) using bovine plasma gamma globulin as standard.

RESULTS AND DISCUSSION Retinoic acid at a concentration of 10 μM increased alkaline phosphatase activity in both normal rat prostate cells and in R-3327 tumor cells in culture (Table 1). In some clones of the R-3327 tumor, the magnitude of increase over constitutive levels approached 20-fold during a 3 day treatment period. Cell line UMS-1541 (7) which is an exceptionally rapidly growing cell derived from a R-3327 tumor, also exhibited some inducibility. This line is of particular interest, however, because one of its clones, UMS-1541Q, exhibits very low constitutive levels of the enzyme yet is strongly inducible by retinoic acid. This clone has also subsequently been found to be tumorigenic. The tumor which is produced by the subcutaneous injection of UMS-1541Q cells into the flank region of an F_1 male hybrid of a

TABLE 1. Effect of Retinoic Acid on the Induction of Alkaline Phosphatase in Cells of Normal Rat Prostate and R-3327 Tumor. All cells were grown in 35 mm culture wells. Values represent the mean of 3 determinations ± S. E.

Cell	Induction Period (Days)	Alkaline Phosphatase Activity	
		Control	Retinoic Acid (10 μM)
Rat Prostate [a]	4	6.7 ± 0.2	13.6 ± 0.7
R-3327G (uncloned) [b]	3	32.5 ± 2.3	72.3 ± 3.5
R-3327G Clones [c]:			
A	3	22.0 ± 3.6	89.5 ± 2.0
D	3	5.2 ± 0.2	52.8 ± 7.2
K	3	7.3 ± 0.8	72.2 ± 12.6
M	3	2.9 ± 0.4	53.7 ± 2.6
UMS-1541 [d]	2	0.5 ± 0.2	11.7 ± 0.7
UMS-1541Q [e]	2	0.0	999.0 ± 45.8

[a] Normal rat prostate cells were obtained by the enzymatic dissociation (2) of a prostate from an F_1 hybrid of a Copenhagen male x Fischer female mating.

[b] Cells were obtained from the outgrowth of explants from an R-3327G tumor at the 26th *in vivo* passage.

[c] Cells for cloning were obtained by the enzymatic dissociation of an R-3327G tumor at the 24th *in vivo* passage. The dispersed cells were subcultured twice before cloning in soft agar (13) in CMRL medium containing 30% fetal calf serum. 2-mercaptoethanol was not included in the medium.

[d] The origin of cell line UMS-1541 has been described previously (7).

[e] Cell line UMS-1541 was cloned in soft agar at the 128th passage.

Copenhagen male x Fischer female mating exhibits high levels of alkaline phosphatase activity. We have obtained values in excess of 3000 nmoles p-nitrophenol liberated/min/mg protein on UMS-1541Q tumor homogenates. When pieces of tumor are explanted to culture and cell cultures established, constitutive enzyme levels decrease over a period of time. In a typical experiment, enzyme levels decreased to 25 nmoles p-nitrophenol liberated/min/mg protein during a 2 month period in culture. Alkaline phosphatase activity increased, however, to 250 nmoles p-nitrophenol liberated/min/mg protein following a 48 hour exposure period to 10 μM retinoic acid.

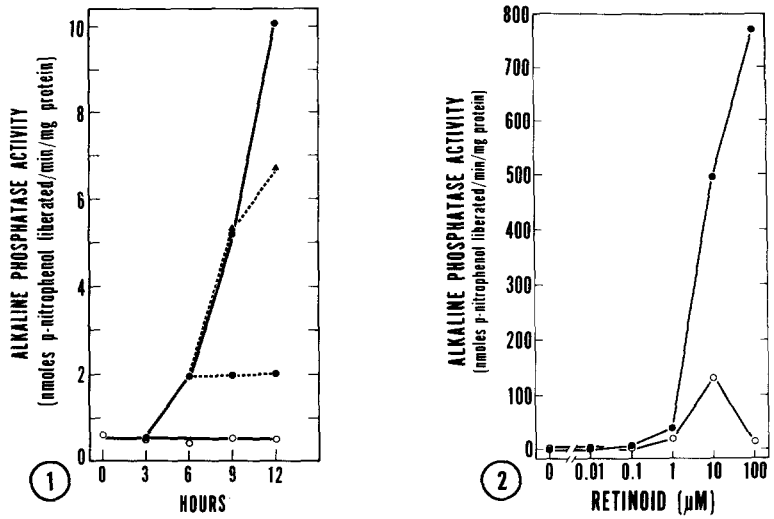

Fig. 1 Time-course of alkaline phosphatase induction by retinoic acid in UMS-1541Q cells. Cells were grown to confluence in 100 x 20 mm culture dishes containing 20 ml of medium. All-trans-retinoic acid (in absolute ethanol) was added at a final concentration of 10 μM. At 6 hours some retinoic acid-treated cultures also received either cycloheximide (20 μM) or actinomycin D (2 μg/ml). Control (0—0); retinoic acid (●—●); retinoic acid + cycloheximide (●--●); retinoic acid + actinomycin D (▲--▲).

Fig. 2 Dose-response for the induction of alkaline phosphatase in UMS-1541Q cells by retinoic acid and retinol. Cells were seeded into 35 mm culture wells at a density of 1 x 10^5 cells/well. After 24 hours, the medium was replaced with fresh medium containing either retinoic acid or retinol at the indicated concentration. Stock solutions were prepared in dimethyl sulfoxide. Cultures were grown for an additional 48 hours at which time the monolayers were processed for alkaline phosphatase and protein measurements. Retinoic acid (●—●); retinol (0—0).

Increased alkaline phosphatase activity can be easily detected in UMS-1541Q cells in culture between 3 and 6 hours after the addition of 10 μM retinoic acid (Figure 1). Using dimethyl sulfoxide instead of ethanol as a vehicle for retinoic acid we have been able to detect increased enzyme activity as early as 3 hours after addition (data not shown). If cycloheximide is added 6 hours after the addition of retinoic acid, the increase in alkaline phosphatase activity is immediately blocked (Figure 1). If, on the other hand, actinomycin D is added at 6 hours, the rate of increase proceeds normally for the next 3 hours then declines during the subsequent 3 hour period. This observation suggested that sufficient mRNA (either for the synthesis of new enzyme

or a regulator of the enzyme) was made during the first 6 hours of the induction process to sustain increased alkaline phosphatase activity in the presence of actinomycin D for at least 3 hours. Cycloheximide or actinomycin D added concomitantly with retinoic acid (data not shown) totally blocked enzyme induction. Collectively, these inhibitor studies indicate that new RNA and protein synthesis is necessary for the increased alkaline phosphatase activity induced by retinoic acid. Whether retinoic acid initiates the synthesis of new enzyme or whether it causes an increase in the catalytic efficiency of existing enzyme is not known at this time. The latter possibility has been shown to be the mechanism for the glucocorticoid induction of alkaline phosphatase activity in HeLa cells (8). In addition to the requirement for a different inductor, other significant differences exist between the induction process in HeLa and R-3327 tumor cells, however. Induction in HeLa cells occurs only after a significant lag period of 12-20 hours. This lag period has been attributed to a requirement for prior DNA replication before catalytically-efficient enzyme can be produced (8). In UMS-1541Q cells, the lag period is on the order of 3 hours and DNA replication does not appear to be a significant factor inasmuch as these experiments were performed on heavily-confluent, stationary cell cultures. The question of whether retinoic acid initiates new enzyme synthesis or catalytic activation of existing enzyme is currently being investigated.

Compared to retinoic acid, its metabolic precursor, retinol, was ineffective as an inducer of alkaline phosphatase (Figure 2). Retinol also caused complete cell killing at 100 μM while retinoic acid at this concentration exhibited no visible signs of cytotoxicity in the form of cell sloughing. The total protein content per dish from cultures treated with 100 μM retinoic acid was only half the value of cultures exposed to lower concentrations, however, suggesting that growth was inhibited at this extreme concentration. Retinal (vitamin A aldehyde) at 10 μM increased enzyme activity equivalent to that of 10 μM retinol (data not shown). Induction of alkaline phosphatase activity by retinoic acid could be detected between 0.1 and 1.0 μM but the most effi-

cient induction occurred above 1.0 μM. These comparisons between retinoic acid and retinol are consistent with those of a previous study (9) which showed that retinoic acid was more effective than retinol in suppressing methylcholanthrene-induced hyperplasia in prostatic organ cultures.

In spite of considerable interest in recent years in the action of vitamin A and its analogs (collectively referred to as retinoids), their mechanism of action, with the exception of the visual process, is still not understood. This is due, in large part, to the absence of a marker or specific gene product for retinoid action. The RNA and protein synthesis-dependent induction of alkaline phosphatase activity in prostatic tumor cells now provides a rapid and easily measurable marker for the action of retinoids. Our observation that increased enzyme activity can be detected as early as 3 hours after the addition of retinoic acid and that this increase can be blocked by inhibitors of RNA and protein synthesis demonstrate similarities to other systems in which steroid hormones bring about the synthesis of specific gene products (10). These similarities, combined with the demonstrations of specific vitamin A-binding proteins in various tissues (11), provide support for the suggestion that the mechanism of vitamin A action is similar to that of the steroid hormones (12).

ACKNOWLEDGEMENTS

We thank Dr. G. Chader for reviewing this manuscript. Research supported in part by N.I.H. grants SO7 RR 05363-19, 5R26 CA 21696-03, and 5R26 CA 15480-05, and the Urology Research Fund, Inc.

REFERENCES

1. Isaacs, J.T., Heston, W.D.W., Weissman, R.M., Coffey, D.S. (1978) Cancer Res. 38, 4353-4359.
2. Claflin, A.J., McKinney, E.C., Fletcher, M.A. (1977) Oncology 34, 105-109.
3. McComb, R.B., Bowers, G.N., Posen, S. (1970) Alkaline Phosphatase, pp. 883-890, Plenum, New York.
4. Riley, P.A. and Spearman, R.I.C. (1968) Science 160, 1006-1007.
5. Healey, G.M. and Parker, R.C. (1966) J. Cell Biol. 30, 531-539.
6. Bradford, M. (1976) Anal. Biochem. 72, 248-254.
7. Malinin, T.I. and Claflin, A.J. (1979) Models for Prostatic Cancer, pp. 111-114, Alan R. Liss, Inc., New York.
8. Cox, R.P., Ghosh, N.K., Bazzell, K., Griffin, M.J. (1975) Isozymes I: Moleculare Structure, pp. 343-365, Academic Press, New York
9. Lasnitzki, I. and Goodman, D.C. (1974) Cancer Res. 34, 1564-1571.

10. Chan, L., Means, A.R., O'Malley, D.W. (1978) Vitamins and Hormones 36, 259-295.
11. Chytil, F. and Ong, D.E. (1978) Vitamins and Hormones 36, 1-32.
12. Bashor, M.M., Toft, D.O., Chytil, F. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 3483-3487.
13. Hamburger, A.W. (1979) Tissue Culture Assoc. Manual 5, 1141-1145.