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EVIDENCE FOR THE RETINOID CONTROL OF UROTHELIAL ALKALINE PHOSPHATASE

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SUMMARY: The amount of alkaline phosphatase activity per μg of DNA in the urothelium (transitional epithelium) of the rat urinary bladder, organ-cultured in chemically-defined serum-free medium, decreased greater than 70% during a 13 day culture period. This decrease in enzyme activity corresponded inversely with the increase in cell number in the urothelium indicating that enzyme synthesis did not accompany growth. Alkaline phosphatase activity was increased back to values approaching normal enzyme levels during a 3 day culture period by the addition of 10 μM retinoic acid. Retinol also increased enzyme activity but it was only half as effective as retinoic acid. A significant increase in enzyme activity was initiated by 1 μM retinoic acid, however the most effective concentration was at 10 μM.

Cells in the intermediate and basal layers of the urothelium (transitional epithelium) of the mammalian urinary bladder are normally rich in the membrane-bound enzyme alkaline phosphatase (EC. 3.1.3.1) (1). Numerous studies employing a variety of experimental animals and classes of chemical carcinogens have consistently demonstrated that urothelial alkaline phosphatase activity diminishes during bladder carcinogenesis (2-8). In the rat, in which this phenomenon has been characterized in detail (4), enzyme loss has been shown to be multifocal, to occur prior to the appearance of hyperplasia and cytological atypia, to be irreversible, and to correlate with regions of the urothelium in which rapid cell proliferation and tumor development subsequently appear. An analysis of the factors involved in this carcinogen-induced defect in the alkaline phosphatase regulatory mechanism should greatly contribute to our understanding of the complex sequence of events which occur during the malignant process in the bladder. Before we can proceed, however, it is essential that we understand the mechanism by which alkaline phosphatase is normally regulated in urothelial cells. Of the various agents which have been

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proposed as regulators of alkaline phosphatases in different cells and tissues (9), retinoids (vitamin A and its analogs) are of particular interest because they have been shown to be required for normal urothelial differentiation (10,11). Retinyl acetate has been shown to increase alkaline phosphatase activity in mouse skin when applied topically (12) and we have shown recently (13) that retinoic acid causes the rapid, RNA and protein synthesis-dependent induction of alkaline phosphatase activity in normal and malignent rat prostatic cells. In the present paper we have used a chemically-defined organ-culture system developed for the rat urinary bladder to show that retinoids are the probable regulators of urothelial alkaline phosphatase activity.

MATERIALS AND METHODS Whole bladders from male Fischer 344 rats (Charles River Breeding Lab.) at 8 weeks of age were cultured in serum-free Ham's F12 medium as described previously (14, 15) Medium was changed at 2-3 day intervals. Stock solutions of all-trans-retinoic acid and all-trans-retinol (Sigma Chemical Co.) were prepared in absolute ethanol redistilled from NaOH or in dimethyl sulfoxide. The final concentration of vehicle in all media was 0.1%. For the analysis of alkaline phosphatase, the urothelium was scraped with a scalpel from its underlying stroma into 1 ml of 0.2% Nonidet P40 (Particle Data Lab.) lysing medium containing 1 mM $MgCl_2$. We have previously shown that the urothelium is quantitatively removed by the scraping technique (14, 16). The samples were rigorously vortexed for 1 min at room temperature, frozen at -20°, thawed, and re-vortexed an additional min prior to analysis. The small amount of blood in the capillary network of normal (0 day) bladders was extruded prior to scraping by grasping the unopened bladder at the dome with forceps and applying pressure with a flat instrument beginning at the dome and continuing toward the cut surface. Aliquots of urothelial lysates were incubated at 37° in 0.7 M 2-amino-2-methyl-1-propanol buffer (pH 10.3) containing 1 mM $MgCl_2$ and 16 mM p-nitrophenylphosphate as substrate. Reactions were stopped with NaOH and the amount of p-nitrophenol liberated was measured spectrophotometrically at 410 nm. DNA was determined by the diphenylamine method (17).

RESULTS AND DISCUSSION The change in urothelial alkaline phosphatase activity was followed over a 13 day culture period using the same culture conditions which we have previously shown cause hyperplasia, dysplasia, and a pattern of growth which closely mimics the growth pattern which is observed during the preneoplastic phase of experimentally-induced bladder cancer (15, 18). The total amount of alkaline phosphatase activity in the urothelium decreased about 30% during this culture period (Figure 1). Although urothelia become hyperplastic in culture which implies an abnormal accumulation of cells on the urothelium, some cells are nevertheless sloughed into the medium. It is

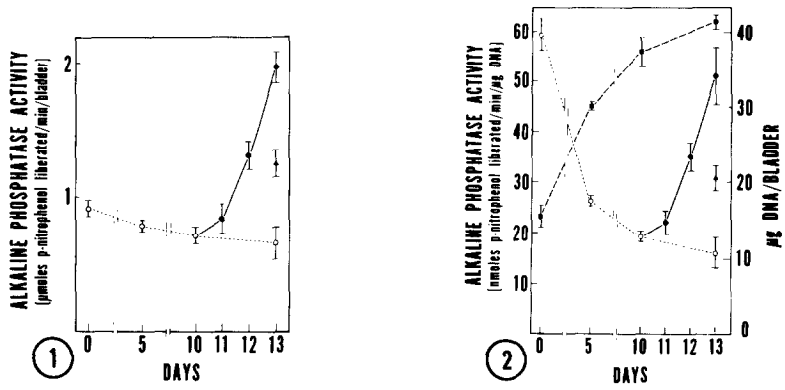

Fig. 1 Time-course of loss of alkaline phosphatase activity and its reinduction by retinoic acid and retinol in organ-cultured urothelia. After 10 days of culture, some bladders were transferred to medium containing either 10 μM all-trans-retinoic acid or 10 μM all-trans-retinol. All points represent the mean of determinations on 4 bladders ± S.E. Alkaline phosphatase activity: in the absence of retinoid (o----o); in the presence of 10 μM retinoic acid (●—●); in the presence of 10 μM retinol (▲).

Fig. 2 Time-course of loss of alkaline phosphatase activity and its reinduction by retinoic acid and retinol as a function of cell proliferation. Details and symbols are the same as Fig. 1. μg DNA/bladder (■——■).

probably this cell sloughing as well as some protein turnover which account for the decrease in total alkaline phosphatase activity. Based on the considerable amount of enzyme remaining in the urothelium, however, the majority of cells appear to remain the the urothelium. If retinoic acid was added at a concentration of 10 μM between day 10 and 13 of the culture period (Figure 1), a dramatic increase in alkaline phosphatase activity was observed. Enzyme activity was increased to values well above that observed in normal (0 day) urothelia. If we take into consideration that extensive cell proliferation occured during the culture period, however, the amount of induction expressed on a per cell (or DNA) basis would not be expected to be as dramatic. This can, in fact, be seen in Figure 2. The DNA content per bladder increased 2-3-fold during the culture period. Consequently the amount of alkaline phosphatase activity per μg of DNA decreased greater than 70% during the culture period. These data indicate that little or no active enzyme was produced during the culture period. Retinoic acid added to the medium at day 10 at a

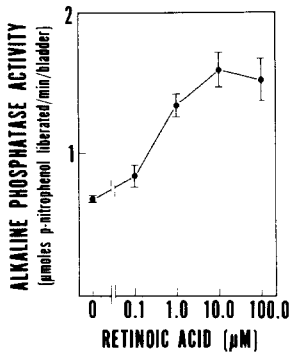

Fig. 3 Dose-response for the induction of alkaline phosphatase activity by retinoic acid. Bladders were cultured for 10 days in the absence of retinoid and then transferred to medium containing retinoic acid at the indicated concentration for 3 days prior to analysis for alkaline phosphatase activity. Retinoic acid stock solutions were prepared in dimethyl sulfoxide.

concentration of 10 μM reestablished enzyme activity over a 3 day period back to values approaching that of normal urothelia. Retinol increased enzyme activity, however, we have consistently observed it to be only half as effective as retinoic acid in the 10 μM range (Figure 2). A similar observation has been made recently on prostatic tumor cells in culture (13), however, relative to retinoic acid, retinol appears to be more effective as an inducer of alkaline phosphatase in the urothelium than in prostatic tumor cells.

The most effective induction of alkaline phosphatase by retinoic acid occured at a concentration of 10 μM (Figure 3). It can be seen, however, that induction could be detected at 0.1 μM and a significant increase in enzyme activity was observed at 1.0 μM. It is interesting that in our previous study on prostatic tumor cells (13) only slight induction could be detected at these lower concentrations. These observations and the above observation that retinol is a more effective inducer in the urothelium than in prostatic tumor cells indicate that the urothelium may be more responsive to retinoids than prostatic tumor cells.

This study and the previous study (13) raise many questions. For example, it is not yet clear whether retinoic acid induces the synthesis of new alkaline phosphatase or whether it induces the synthesis of a protein which catalytically

activates existing inactive enzyme. This latter possibility has been shown to be the mechanism by which glucocorticoids induce alkaline phosphatase in HeLa cells (19). Also, the characterization of the chemical properties of native and retinoic acid-induced enzyme has not been completed and thus we do not know, at this time, if induced and native alkaline phosphatases are the same isozyme. We think, however, that inasmuch as vitamin A is known to be required for the maintenance of normal urothelial differentiation, retinoic acid (or a metabolite) is likely to be the natural regulator of the urothelial enzyme.

In recent years, evidence has accumulated which suggests that the action of retinoids is mediated, as it is in the case of steroid hormones, by cytoplasmic receptor proteins which specifically bind retinoids (20). Once bound, the retinoid+receptor complex is thought to be translocated to the nucleus where it initiates the synthesis of specific gene products. Carcinogen damage to the alkaline phosphatase regulatory mechanism in the urothelium could occur at any point in this sequence. The identification of retinoic acid as the probable regulator now provides a framework in which to carry out studies aimed at identifying the site(s) of carcinogen damage to the alkaline phosphatase regulatory system in the urothelium.

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