Inhibition of Transformation of Primary Rat Tracheal Epithelial Cells by Retinoic Acid

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ABSTRACT

The effect of retinoic acid (RA) on N-methyl-N'-nitro-N-nitrosoguanidine-induced transformation of primary cultures of rat tracheal epithelial cells was investigated. RA inhibited transformation of rat tracheal epithelial cells by up to 95% at concentrations of 3.3 to 33 μM which did not substantially affect cell survival. The inhibitory effect of RA on transformation was concentration dependent and was also dependent upon timing and duration of treatment. Treatment with RA for only 1 week following N-methyl-N'-nitro-N-nitrosoguanidine exposure diminished the transformation frequency by 30 to 57%, although longer treatment times were more effective. Because RA was able to inhibit transformation effectively at concentrations which were not substantially inhibitory to colony-forming efficiency of rat tracheal epithelial cells, the mechanism of inhibition of cell transformation does not seem to be related to cytotoxic effects of RA known to occur at high RA concentrations.

INTRODUCTION

The possibility of chemoprevention of cancer has intrigued many experimentalists. Up to the present, 2 strategies have been considered: (a) pharmacological or nutritional intervention with the goal of preventing the interaction of toxic chemicals or their reactive carcinogenic intermediates with macromolecular targets; and (b) inhibition of promotion and progression of preneoplastic stages (11, 20, 21). Attempts to prevent tumor development by administration of retinoids (vitamin A analogues) belong to the latter category. Inhibition of carcinogenesis by retinoids has been convincingly demonstrated in mouse skin tumor initiation-promotion studies (25), in rat mammary carcinogenesis studies (5), and in mouse and rat bladder carcinogenesis studies (1, 22). Most attempts to inhibit respiratory tract carcinogenesis in either rats or hamsters by administrations of high doses of retinoids have failed (18, 19, 23, 26), but some encouraging results have been reported (17).

Several investigators have used organ and cell culture systems to study the cellular effects of retinoids and have demonstrated inhibition of cell division in carcinogen-treated prostate organ cultures (2, 6), inhibition of growth of neoplastic cell lines (7), reversal (or inhibition) of carcinogen-induced metaplastic changes of tracheal epithelium (10), and inhibition of in vitro transformation of C3H10T1/2 mouse embryo fibroblasts (8, 9).

Our laboratory has been involved in studies concerned with the modulation of neoplastic development by retinoids in respiratory tract epithelium. These studies have shown that vitamin A-deficient rats have an increased susceptibility to lung cancer induction (12-15), but inhibition of respiratory carcinogenesis in rats or hamsters by pharmacological doses of retinoids in animals maintained on adequate levels of vitamin A was not observed (14, 15, 26). While these studies indicate that retinoids can modulate carcinogenesis of airway epithelium, they do not permit examination of the cellular and biochemical mechanisms of this effect.

Recently, we succeeded in establishing a quantitative clonal transformation assay using rat tracheal epithelium (24) and have begun to use this cell culture transformation system to investigate the effects of retinoids in epithelial carcinogenesis. In this transformation system, tracheal epithelial cells, isolated from normal adult rats, are seeded at clonal densities on either collagen (16) or on monolayers of 3T3 feeder cells (4, 24) and are exposed 24 hr later to a carcinogen, such as MNNG. Three to 5 weeks after such an exposure, foci containing densely packed, mitotically active, atypical epithelial cells appear in exposed cultures. These cultures have markedly enhanced capacity for survival. The EG variants which frequently become immortal are considered to be RTE cell transformants and have been shown to give rise to neoplastic cells after further growth in culture (16, 24).

In this preliminary paper, we report that RA reproducibly inhibits in vitro transformation of RTE cells induced by carcinogen exposure. This inhibitory effect is dependent on RA dose and the timing of RA treatment.

MATERIALS AND METHODS

The preparation of primary RTE cells has been described previously (4). The essential details will be recounted here. Primary tracheal cells from 8-week-old male Fischer 344 specific-pathogen-free rats were obtained by protease treatment of isolated tracheae. Suspensions of single RTE cells were seeded onto monolayers of lethally irradiated 3T3 cells. RTE cells were allowed to attach for 24 hr at 37° in a humidified 95% air-5% CO₂ atmosphere. The culture medium was Ham’s F-12 containing 5% fetal bovine serum (Lots 29K5209 and 31K9626; Grand Island Biological Co., Grand Island, NY), insulin (1 μg/ml), and hydrocortisone (0.1 μg/ml). Gentamicin (100 units/ml) and streptomycin (100 μg/ml) were used as antibiotics. Cultures were exposed for 4 hr to MNNG dissolved in Ham’s F-12 medium, lacking serum, but including a final concentration of 20 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid at pH 6.8. RA (all-trans; Sigma Chemical Co., St. Louis, MO) was dissolved in dimethyl sulfoxide (silylation grade; Pierce Chemical Co., Rockford, IL) at a concentration of 1 mg/ml and was stored frozen under liquid N₂. At the time RA was needed, an aliquot was thawed from liquid N₂ under subdued light and added to the culture medium. Culture medium containing RA was dispensed within 30 min of dilution from stock. In all
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experiments, the culture medium supporting RTE cells was replaced 2 times per week. In some experiments, 3T3 cell monolayers were removed from tracheal cell cultures 1 week after carcinogen exposure using 0.002% EDTA (24). After a total of 5 or 6 weeks in culture, cells on culture dishes were fixed with absolute methanol and stained with aqueous 10% Giemsa.

RESULTS

The RTE cell assay for detection of transformation consists of 2 sequential phases: an expression period, usually ending 6 days after the termination of carcinogen exposure, which allows carcinogen-induced changes to become established; and a selection period which allows growth of only the transformed cells. Selection against the survival of normal RTE cells is accomplished by removing the 3T3 cell monolayer. Foci of carcinogen-altered tracheal cells, termed EG variant colonies, survive the growth restriction imposed by removal of the 3T3 cells and are scored 4 to 6 weeks after carcinogen exposure.

Table 1 summarizes the results of 3 separate experiments in which the RTE cell cultures were treated with RA immediately after exposure to MNNG. There are 4 groups in each experiment: Group 1, cultures that received neither carcinogen exposure nor retinoid exposure but received suspending vehicle solvent; Group 2, cultures that were exposed to MNNG only; Group 3, cultures that were exposed to MNNG and were subsequently treated with RA; and Group 4, cultures that received RA. After 7 days, 5 cultures from each of the 4 groups were removed from the experiment and stained in order to calculate cytotoxicity of the various exposures based upon changes in CFE. In Experiments 1 and 2, an initiating dose of MNNG of 0.3 \( \mu \text{g/ml} \) was used (see Table 1). Continuous RA exposure (33 nM) was begun immediately after the 4-hr MNNG exposure. The MNNG exposure reduced CFE of RTE cells by about 50%. RA alone did not significantly affect CFE. No reduction of colony size in RA-treated cultures was noted. After sequential exposure to MNNG and RA, the CFE was not significantly lower than after exposure to MNNG alone in Experiment 1 but was reduced by 32% in Experiment 2. MNNG exposure produced transformation fre-
RA INHIBITION OF RTE CELL TRANSFORMATION

Chart 1. Concentration-dependent inhibition of RTE cell transformation by RA. The initial number of RTE cells seeded per 60-mm dish was 5000; exposure to MNNG (0.3 μg/ml) was used to induce transformation. Exposure to RA began immediately after MNNG was removed.

% INHIBITION OF TRANSFORMATION

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<th>Duration of RA Exposure (WEEKS)</th>
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DURATION OF RA EXPOSURE (WEEKS)

Chart 2. Effect of timing and duration of RA treatment (33 nM) on MNNG-induced transformation frequency. RTE cells (5000/dish) were exposed to MNNG (0.3 μg/ml) to induce transformation. Shaded bars, duration of exposure to RA. (49 to 57% inhibition of transformation), but it was less inhibitory than the full 6-week treatment.

DISCUSSION

These studies presented here demonstrate that RA markedly inhibits the development of RTE cell transformation in cultures treated with RA after exposure to the direct-acting carcinogen MNNG. This suppressive effect was dependent on RA concentration. About 95% inhibition of transformation frequency was obtained with 165 to 330 nM RA and 65% with 16 to 33 nM RA (see Chart 1). In the experiment in which a different serum lot was used, 80% suppression of transformation was observed with as little as 3.3 nM RA (see Table 1). It is important to note that the inhibition of transformation occurred in these experiments at RA concentrations that caused only little inhibition of CFE for RTE cells. Thus, the observed effect cannot be explained simply by overt retinoid toxicity for RTE cells. This raises the possibility that transformed RTE cells are more susceptible to the toxic effects of RA than untransformed cells. However, ongoing studies with several transformed RTE cell lines do not support such an interpretation.

In one series of experiments, the time as well as the duration of RA treatment during the development of transformed colonies was varied (see Chart 2). Significant inhibition of transformation occurred even when RA treatment lasted only for 1 week. Inhibition of transformation was also observed when RA treatment was delayed and was started as late as 2 weeks after carcinogen exposure. This latter finding suggests that the observed effect is not due to modulation of early transformation events, such as carcinogen metabolism (in the case of MNNG, mechanisms accelerating decomposition and detoxification), DNA damage and repair, "fixation," and expression of molecular lesions leading to transformation. Rather, RA treatment seems to interfere with mechanisms related to a later phase of transformation which is concerned with the formation of transformed colonies. Since the major phenotypic change of RTE cell transformants is EG, we suspect that RA may act by interfering with this abnormal growth capacity.

Our findings are consistent with observations reported previously by Merriman and Bertram (8) on the inhibition by retinoids of transformation in C3H10T½ mouse embryo fibroblasts. These investigators found that retinyl acetate as well as retinal and retinol inhibit chemically induced transformation in a dose-dependent manner. Only 1 week of treatment was required to effect inhibition of transformation, and the start of treatment could be delayed for 3 weeks without loss of the inhibitory effect. Interestingly, the authors also found that the inhibition of transformation by retinoids was reversible, suggesting that the progression of initiated cells to transformed cells was arrested (8) rather than the state of initiation being affected. With the information presently available, it is not possible to determine whether RA inhibits the phenotypic expression of transformed RTE cells or whether it inhibits progression from the initiated to the transformed state. To be able to make this important distinction will require further experimentation.

The mechanism of inhibition of cell transformation by retinoids is presently unknown. That it occurs in such different cell systems as a mouse fibroblast cell line and primary RTE cell cultures suggests that rather basic mechanisms are involved. Particularly intriguing is the fact that, while in vivo administration of high levels of retinoids does not inhibit the development of respiratory tract tumors in rats, vitamin A deficiency does increase respiratory tract tumor incidence. This increased susceptibility to tumor induction is abolished by reintroducing retinoids into the diet (12–15). It is conceivable that the RTE cell culture system is relatively vitamin A deficient and that this contributes to the rather high
transformation frequency that can be induced with chemical carcinogens (24). Cell culture models, such as the C3H10T\(\text{1/2}\) (8) and the RTE cell transformation system, seem to be well suited to unravel some of the complex questions raised by the in vivo studies on the antineoplastic effects of retinoids (1, 5, 17, 21, 22, 25). The RTE cell system has the added advantage that a well-defined in vivo counterpart is available to study progression and modulation of neoplastic transformation in vivo (13). Hopefully, the cell culture models will also help to explain why retinoids are effective inhibitors of carcinogenesis in some but not in other tumor induction models.

REFERENCES

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