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Modulation of 3-methylcholanthrene toxicity in cultured neoplastic keratinocytes by glucocorticoids and retinoids is not accounted for by macromolecular adduct formation

(differentiation/polycyclic aromatic hydrocarbons/bioactivation)

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ABSTRACT 3-Methylcholanthrene (3-MC) greatly inhibits the growth of two lines of human squamous carcinoma cells, SCC-9 and SCC-12B2. Exposure of the cells to 2,3,7,8-tetrachlorodibenzo-p-dioxin alone was much less effective and, in the presence of 3-MC, did not alter the sensitivity (EC50 = 0.3 μM) or extent of growth inhibition by the latter. The degree of 3-MC-mediated inhibition, however, was markedly alleviated by inclusion of retinoic acid (EC50 ≥ 0.7 μM) and hydrocortisone (EC50 = 40 nM) or dexamethasone (EC50 = 3 nM) in the culture medium. These physiological effectors, which are known to have opposing actions on keratinocyte character in SCC cells, did not significantly alter either aryl hydrocarbon hydroxylase activity or macromolecular adduct formation. Further analysis of the cellular responses indicated that hydrocortisone and, in some experiments, retinoids increased the growth rate in 3-MC-exposed cultures, while 3-MC increased the saturation density in retinoic acid-exposed cultures, an example of interference with a physiological response of the cells. These results indicate that alteration of the differentiated state, regardless of the direction of the change, can alter the sensitivity of these cells to toxic stimuli. Further investigation of the bases of such toxic responses and their modulation by the microenvironment may enhance our understanding of the target cell specificity of polycyclic aromatic hydrocarbons.

The influence of toxicity from chemical carcinogens on the generation of malignancy is difficult to predict a priori, but it likely plays a major role in some instances. For example, the relationship seems clear in the liver, where development of hepatocarcinoma in response to diethylnitrosamine exposure is promoted at high doses by cell death (necrosis) and generation of resistant hepatocyte populations (1). However, in at least one cell culture system, cytotoxicity and malignant transformation by carcinogens can be separated (2). Thus, treatment with dimethylbenzanthracene strongly inhibited survival and colony-forming efficiency of the embryonic mouse line C3H/10T1/2 in parallel with a stimulation of transformation. This relationship, however, was not obtained either for the closely related compound 3-methylcholanthrene (3-MC), which stimulated transformation with only a moderate inhibition of growth and no effect on plating efficiency, or N-methyl-N'-nitro-N-nitrosoguanidine, which was very toxic but had no transforming effect. Similarly, the α-naphthoflavone-induced reduction of DNA binding, mutagenesis, and cytotoxicity by aflatoxin B1 in this system was not accompanied by a reduction of transformation (3). In some tissues, notably the epidermis (4) and prostate (5), toxicity may be expressed as a proliferative or metaplastic response. Metaplasia often precedes and thus may predispose a tissue to malignant change, though this issue is controversial (6).

Improvements in cell culture methodology over the past decade now permit serial culture of keratinocytes from a variety of tissues and species (7), facilitating study of the interaction of chemical carcinogens with an important target cell type. In one such study polycyclic aromatic hydrocarbon (PAH) compounds induced mutagenesis with minimal toxicity in a clonal keratinocyte line derived from a human squamous cell carcinoma (8). In other work, several keratinocyte lines derived from rat and human tissues were subject to growth inhibition at clonal densities by 3-MC (9). This inhibition was not directly correlated with the induction of carcinogen metabolism, however, since early passage rat esophageal epithelial and epidermal cells showed similar levels of aryl hydrocarbon hydroxylase (AHH) activity, but toxicity was evident only in the latter cultures. In the present study we have characterized the effects of 3-MC on the growth of human squamous carcinoma cells. These cells are useful not only because of their human origin and well-characterized responses to physiological effectors but also because they show a pronounced and easily quantifiable toxic response to 3-MC, an inhibition of the growth rate. The experiments exploit the observation that hydrocortisone (HC) and all-trans-retinoic acid (RA) in the medium largely alleviate the growth inhibitory effect of 3-MC. In light of the reports of possible prophylactic effects of retinoids and glucocorticoids on carcinogenesis both in vivo (10, 11) and in vitro (12–14), this system offers an effective way to analyze the interactions between carcinogens and such important physiological effectors at the cellular level. The present results indicate that HC and RA improve the growth of 3-MC-inhibited cells by pathways that do not involve altered macromolecular adduct formation.

MATERIALS AND METHODS

Cell Culture. Stock cultures of SCC-9 and SCC-12B2 keratinocytes, originally derived from squamous carcinomas of the human tongue and facial epithelms, respectively (15), were maintained with irradiated 3T3 feeder layer support (16). The medium consisted of 3 parts Dulbecco's modified Eagle's medium (DMEM) and 1 part Ham's F12 medium and was supplemented with 0.1 mM adenine, insulin at 5 μg/ml, transferrin at 5 μg/ml, and 20 pM triiodothyronine. The medium for SCC-9 cells was also supplemented with 2% fetal bovine serum (HyClone) during inoculation and 1% fetal

Abbreviations: PAH, polycyclic aromatic hydrocarbon(s); 3-MC, 3-methylcholanthrene; AHH, aryl hydrocarbon hydroxylase; BP, benz(a)pyrene; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; HC, hydrocortisone; RA, all-trans-retinoic acid; EC50, the concentration at which a half-maximal effect is elicited.

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bovine serum in subsequent medium changes. For SCC-12B2 cells (Fig. 1) DMEM was supplemented with 2% retinoid-free solvent-extracted fetal bovine serum (17). In experiments analyzing confluent cultures 0.25–1.0 × 10⁵ SCC-9 cells per 6-cm dish were inoculated in the presence of 5 × 10⁵ irradiated 3T3 cells, whereas, in experiments involving counting of cells at low density, the 3T3 feeder cells (unnecessary for short-term maintenance of these cultures) were excluded from the initial plating. Cell numbers were determined electronically. In all of the experiments in which cell numbers were determined, PAH, glucocorticoids, retinoids, or their appropriate vehicle controls (dimethyl sulfoxide, ethanol) were added when the cells were at low density as indicated in each experiment and again with each medium change.

AHH Activity. Low-density cultures (1.4 × 10⁵ SCC-9 cells per dish) were incubated in the presence of glucocorticoid or retinoid for 24 hr before the addition of 10 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which is only weakly toxic to these cells (18) and not significantly metabolized. This approach avoided two problems arising from the use of 3-MC as the inducing stimulus: (i) toxicity evident upon treatment of low-density cultures and (ii) interference with the assay by the high residual concentrations of unlabeled 3-MC required for induction. After 1 week of exposure to TCDD, AHH was assayed in intact cells by measuring the conversion of [³H]benzo[a]pyrene ([³H]BP (New England Nuclear) to polar metabolites (19). Cultures were rinsed four times in serum-free medium and exposed to 12 μM [³H]BP (10 nCi/mmol; 1 Ci = 37 GBq) for 90 min. The unmetabolized BP was removed by three extractions with hexane and 0.5 ml of the alkaline aqueous phase containing the metabolites was neutralized with 2 ml of 1 M Tris-HCl, pH 7.7, before scintillation counting in 15 ml of Aquasol (New England Nuclear) (9). Protein concentration was assayed (20) by using Coomassie brilliant blue G-250 reagent (Bio-Rad).

Measurement of Adduct Formation to Protein and DNA. Logarithmic-phase cells (10–30% confluent) in 10-cm dishes were simultaneously exposed to RA (3.3 μM) and HC (1 μM) or vehicle controls in the presence or absence of 10 nM TCDD. After 1 week the cultures, which had become confluent, were rinsed four times with serum-free medium and exposed to [³H]BP (3 Ci/mmol for DNA adducts, 0.56 Ci/mmol for protein adducts) diluted with unlabeled BP to a final concentration of 2 μM. After 24 hr the cultures were rinsed three times with serum-free medium and either trypsinized or scraped into 1 ml of 10 mM Tris-HCl, pH 7.4/1 mM EDTA on ice and frozen until assay. DNA adducts were measured by using a modification of the technique of Billings et al. (21). The scraped cells were made 1% in SDS, homogenized, exposed to proteinase K (Sigma) at 0.25 mg/ml for 2 hr at 37°C and then to bovine pancreatic ribonuclease A (Boehringer Mannheim) at 0.1 mg/ml at 37°C for 18 hr. Each sample was extracted with an equal volume of liquefied phenol, then with phenol/chloroform/isoamyl alcohol (25: 24:1, vol/vol), and finally with chloroform/isoamyl alcohol (24:1). The aqueous phase was brought to 0.2 M in NaCl and chilled on ice, and 2 vol of cold 95% (vol/vol) ethanol were added. The precipitated DNA was pelleted at 3600 × g, resuspended in 0.25 ml of 0.15 M NaCl/0.015 M sodium citrate, pH 7, and the total DNA concentration was determined by UV absorbance (A260/A280). After addition of 500 μg of herring sperm DNA as a carrier, each sample was sheared by repeated passage through a 22-gauge needle. Three volumes of ice-cold 5% trichloroacetic acid was added and the samples were filtered under reduced pressure on glass fiber filters, which were then placed in Aquasol for measurement of radioactivity. For protein adduct determination the labeled cells were homogenized and extracted twice with ethyl acetate/acetone (2:1, vol/vol) followed by addition of 4 ml of phenol, 3 ml of Tris/EDTA buffer, and 1.5 ml of chloroform. After centrifuging for 15 min at 12,000 × g, the lower phase (and interface) was washed five times with 3 ml of H₂O and the protein was precipitated with 20 μl of 4 M sodium acetate and 5 ml of 100% ethanol. The pellets were recovered by centrifugation at 4000 × g for 5 min and washed three times with 4 ml of 70% ethanol. The precipitated protein was resuspended in 2 M NaOH and dissolved overnight at 37°C. Aliquots were removed for scintillation counting and total protein was determined by using a bicinchoninic acid (Pierce) modification of the Lowry method (22).

RESULTS
As shown in Fig. 1, initial experiments with two lines of malignant human keratinocytes, SCC-12B2 and SCC-9, showed that inclusion of 3-MC (1 μg/ml) in the medium

![Fig. 1](image-url)

(A) Effect of HC (1 μM), RA (3.3 μM), and 3-MC (1 μg/ml) on growth of low-density cultures of SCC-12B2 cells. One day after inoculation of 1000 keratinocytes on an irradiated feeder layer of 3T3 cells in 6-cm dishes, the medium was changed to include 2% solvent-extracted fetal bovine serum in DMEM. HC, RA, and/or the appropriate solvents (ethanol and dimethyl sulfoxide) were added, followed in 5 hr by 3-MC. The medium was changed twice weekly for 22 days with the experimental compounds added at each medium change. The cultures were fixed and stained with rhodamine blue. (B) Effect of RA and 3-MC on the growth of low-density cultures of SCC-9 cells in the presence of HC. Except for the growth medium (1% fetal bovine serum in DMEM/Ham’s F12) the experiment was performed as in A. The cultures were fixed and stained after 17 days.
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substantially inhibited the growth of low-density cultures, nearly preventing clonal expansion. Addition of either HC (1 μM) or RA (3.3 μM) alone had relatively little apparent effect on cell growth with or without added 3-MC. However, upon addition of both HC and RA, growth in the presence of 3-MC approached that in the absence of the PAH. The SCC-9 cell line was chosen for further investigation of this phenomenon.

In initial experiments using higher cell numbers to quantitate the response to culture conditions, the dependence of growth inhibition on 3-MC concentration was examined. Cultures were exposed to this agent for 2 weeks and then harvested and counted. As shown in Fig. 2, growth was maximally inhibited in the range of 0.3-1 μg/ml with an EC50 estimated at 0.07-0.08 μg/ml (0.3 μM). If growth suppression resulted from 3-MC metabolism, the concentration dependence likely would reflect lower metabolite production due either to limited induction of AHH at the lowest concentrations tested (0.03 and 0.1 μg/ml) or to limited availability of the PAH substrate. To distinguish between these alternatives, the same type of experiment was performed with cultures pretreated with TCDD, which is an effective inducer of AHH in these (23) and other cell types at very low concentration (1-10 nM) without interfering with metabolism or being appreciably metabolized itself (4). In this case, as is evident in Fig. 2, TCDD reduced the cell number slightly, but the dependence of growth inhibition on 3-MC concentration was nearly identical. Thus, the EC50 is unlikely to reflect simply a limitation of AHH activity.

The hypothesis that HC and RA alleviation of growth inhibition by 3-MC reflects modulation of AHH activity was investigated. To this end, cultures growing with or without HC and RA in the medium were examined for their metabolism of BP during a 1.5-hr period. AHH activity was stimulated by 10 nM TCDD in half the cultures. Those cultures without TCDD stimulation approximated basal level metabolism, much as appreciable induction requires several hours of PAH exposure (9). In either case, HC and RA alone or together had essentially no effect on the degree of AHH inducibility or the level of activity attained (Fig. 3).

The possibility that HC and RA alleviated the inhibition of growth by 3-MC through reduction of macromolecular adduct formation was investigated. Cultures grown in the presence or absence of HC and RA were treated for 1 day with [3H]BP and the degree of protein and DNA adduct formation was assessed. As shown in Table 1, both types of adducts were formed only to a low extent. For example, the observed level of protein damage corresponds to ~0.33 adduct per protein of 50 kDa. The degree of DNA labeling, 1% as great on a weight basis and corresponding to 0.2 adduct per million base pairs, was two orders of magnitude lower than that observed in other cells, such as from rat mammary gland (24), found to be sensitive to growth inhibition by PAH. Similar results were obtained when the cells were grown in the presence of 10 nM TCDD. In either case, no clear and consistent reduction in adduct formation was manifest in cells cultured with HC and RA, and hence this explanation could not be invoked convincingly to explain the improvement in cellular growth.

To provide more quantitative information regarding RA and HC modulation of 3-MC responses, the growth rates and saturation densities of cultures were measured under the different culture conditions. As seen in Fig. 4A, inclusion of 3-MC (1 μg/ml) in the medium increased the doubling time of control cultures from approximately 1.5 to 2 days. Fig. 4B illustrates that inclusion of RA (3.3 μM) in the medium had only a minor effect on growth rates in the presence or absence of the PAH compound. However, addition of the retinoid resulted in a substantial reduction in saturation density compared to control cultures grown in its absence. This action and its suppression by 3-MC account for the similar final cell numbers seen under these conditions by about 10 days after inoculation. Examination of the RA-treated cultures after they reached confluence revealed considerable shedding of cells into the medium in the absence but not the presence of 3-MC. In contrast, HC significantly improved the cell growth in the presence of 3-MC, but the cells did not reach the saturation density observed in the absence of the PAH compound (Fig. 4C). (The decreased level of 3-MC-generated toxicity observed here compared to that shown in Fig. 1B may be due in part to the effect of treating

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**Fig. 2.** Concentration dependence of SCC-9 growth inhibition by 3-MC. One day after inoculation the medium was adjusted to 0 (○) or 10 nM (□) TCDD. Two days later, 3-MC was added to give the concentrations indicated. The cells were grown under these conditions for an additional 9 days, at which time they were trypsinized and counted. Error bars represent the range of duplicate determinations.

**Fig. 3.** Effect of culture conditions on AHH activity in SCC-9 cells. Exponential cultures were exposed for 1 day to RA and/or HC with subsequent exposure to 0 (stippled bars) or 10 nM (hatched bars) TCDD for an additional 1 week. At that time they were exposed to 12 μM [3H]BP for 1.5 hr and the degree of conversion to polar compounds was determined. Error bars represent the range of duplicate determinations.

<table>
<thead>
<tr>
<th>Medium additions</th>
<th>Protein adducts, pmol/mg of protein</th>
<th>DNA adducts, pmol/mg of DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>54, 56</td>
<td>0.15, 0.42</td>
</tr>
<tr>
<td>HC + RA</td>
<td>54, 64</td>
<td>0.15, 0.18</td>
</tr>
<tr>
<td>TCDD</td>
<td>76, 74</td>
<td>0.13, 0.11</td>
</tr>
<tr>
<td>HC + RA + TCDD</td>
<td>53, 51</td>
<td>0.11, 0.14</td>
</tr>
</tbody>
</table>

Results are given for duplicate cultures analyzed in parallel.
The present results reveal a substantial suppression of growth by 3-MC in cultures of SCC-9 malignant keratinocytes and a striking alleviation of this action by HC and RA. This suppression might be attributed to the production of 3-MC metabolites, but the evidence regarding its alleviation by HC and RA provides scant support for reduction of AHH activity or of macromolecular adduct formation. Indeed, examination of the cell growth responses to the different culture conditions indicates that the influences of the latter two agents were not equivalent, implying that separate pathways were involved. In addition, AHH-inducing concentrations of TCDD did not alter either the sensitivity or the extent of 3-MC-mediated growth inhibition.

In support of these findings, experiments performed with organ cultures of mouse prostate (26) have shown that RA prevents the hyperplastic effects both of 3-MC and of the direct-acting carcinogen N-methyl-N'-nitro-N-nitrosoguanidine. Our data emphasize the importance of the physiological state of the target as influenced by its hormonal and vitamin status, which in this case has a major influence on the differentiation of SCC lines (25, 27, 28). It appears here that regardless of the apparent direction of the differentiating stimulus (glucocorticoids favor and retinoids counter the expression of keratinocyte properties), the cells were rendered less sensitive to the growth-inhibitory effects of the carcinogen. This might be viewed as an extension to the cellular level of the major influence of development on the expression of malignancy (29).

These conclusions are highly compatible with our recent demonstration of a reverse phenomenon: suppression by PAH compounds of the retinoid-mediated induction of tissue transglutaminase in SCC-4 cells in a manner also apparently independent of biotransformation (30). Similar suppressions by various carcinogens of hormonal inductions have been observed in intact rat liver (31), in rat hepatocytes (33), and in bovine adrenocortical cells (34). Thus, different carcinogens may induce analogous states of hormone insensitivity by "physiological" pathways that do not necessarily involve toxic macromolecular damage. Interestingly, TCDD, which

**DISCUSSION**

The present results reveal a substantial suppression of growth by 3-MC in cultures of SCC-9 malignant keratinocytes and a

**FIG. 4.** Growth of SCC-9 cells in the presence and absence of HC, RA, and 3-MC. Cells were plated at $10^5$ per 6-cm dish without 3T3 feeder cells and exposed to these compounds 2 days later. Virtually identical results were obtained in experiments employing feeder layers, but measurements of SCC-9 cells at early time points were less accurate due to unavoidable contamination by 3T3. (A) Controls; (B) RA; (C) HC; (D) RA + HC. *No 3-MC; ○, 3-MC. All effector concentrations were the same as in Fig. 1. Error bars represent the range of duplicate determinations.

much higher numbers of cells. The interaction of RA and HC, illustrated in Fig. 4D, can be accounted for to a considerable extent by the phenomena observed for each alone. Thus, the growth rate of the cells was similar with or without 3-MC in the medium, due primarily to the action of HC and partially to RA. After confluence, likely due to PAH inhibition of RA-induced shedding, the final cell density in the presence of 3-MC was equal to or even exceeded that in its absence.

**Fig. 5A** shows the dependence of cell growth on glucocorticoid concentration in the presence of 3-MC and RA. The substantial action of HC in antagonizing the growth suppression by 3-MC appears specific for glucocorticoids, inasmuch as dexamethasone was also effective but steroids such as 17β-estradiol and testosterone were inactive. Consistent with previous observations on stimulation of keratinocyte differentiation (25), dexamethasone is the more effective glucocorticoid with respect to its potency (EC$_{50}$ of $\approx 3$ nM vs. 40 nM for HC) and power at maximal concentrations employed (1 µM). Fig. 5B shows the dependence of cell growth on retinoids in the presence of 3-MC and HC. Consistent with Fig. 1, the addition of either retinyl acetate or RA in the micromolar range (EC$_{50}$ $\approx 0.7$ µM) resulted in noticeably improved growth. (In this experiment the degree of 3-MC-mediated inhibition was greater and hence the retinoid effect was more obvious than in Fig. 4 C and D.) This experiment, like Fig. 1, shows that both retinoids and glucocorticoids can alleviate toxicity by promoting the growth of 3-MC-treated cells.


**DISCUSSION**

The present results reveal a substantial suppression of growth by 3-MC in cultures of SCC-9 malignant keratinocytes and a

**FIG. 5.** Dependence of 3-MC-treated SCC-9 cell density on glucocorticoid and retinoid concentrations. (A) Glucocorticoids. Cells were inoculated at $2.5 \times 10^4$ per 6-cm dish and exposed to 3-MC at 1 µg/ml and RA at 3.3 µM in the presence of the indicated concentrations of dexamethasone (●) or HC (○) for 10 further days. As a test of glucocorticoid specificity, separate 3-MC-treated cultures were exposed to 1 µM testosterone (△) or 1 µM 17β-estradiol (▲). (B) Retinoids. Cells were inoculated at $10^5$ per dish and exposed to 3-MC and 1 µM HC in the presence of the indicated concentrations of retinyl acetate (●) or RA (○) for 17 (retinyl acetate) or 15 (RA) further days. Error bars represent the range of duplicate determinations.
is not known to be significantly metabolized or to form adducts, inhibits SCC-13 cell growth in the absence but not the presence of HC while suppressing HC-mediated differentiation (18).

Although retinoids have been reported to interfere with PAH metabolism (35, 36) and to reduce DNA adduct formation by PAH compounds in cell types such as mouse keratinocytes (37), the present results emphasize another aspect of retinoid–PAH interactions meriting attention. A critical action of 3-MC in SCC-9 cells is to increase the cellular saturation density by interfering with the response to a physiological effector in their microenvironment. Our observation of RA-induced shedding of SCC-9 cells is consistent with retinoid promotion of desquamation of normal human epidermal cells in culture (38) and provides a likely site of intervention by PAH contributing to increased saturation density. In addition, as shown in Figs. 1 and 5B, retinoid stimulation of growth in 3-MC-inhibited cells is apparent when the initial level of inhibition is great enough.

HC and RA could conceivably reduce PAH toxicity by altering the spectrum of metabolites produced without changing the total level of biotransformation measured with BP. More plausible, however, is the alteration of intracellular detoxification of the reactive species generated. For example, PAH metabolism is known to produce hydroxylated derivatives that spontaneously autoxidize, yielding toxic free radicals and quinones (39). A recent study of the effects of aromatic hydrocarbons on cultured mammary cells showed that the major determinant of toxicity was the level of reactive oxygen species generated and not the covalent adducts produced (40). Thus, glucocorticoid action in alleviating 3-MC toxicity could arise from stimulating the cellular defense against such compounds. For instance, a variety of agents have been found to induce cytosolic quinone reductase in murine hepatoma cells (41). As exemplified by the level of glutathione (42), a major cellular antioxidant, alteration of the cellular defense may not be reflected in the degree of DNA adduct formation by PAH metabolites. This hypothetical action of HC could offer protection regardless of whether radicals were generated by AHH or through a proposed membrane perturbation (43), and it is consistent with HC suppression of radiation transformation noted with certain cultured cells (14). Similarly, possible anti-oxidant action of retinoids and related compounds such as β-carotene (44) might also occur.

For many carcinogens, our understanding of the determinants of target tissue and species specificity is rudimentary.

PAH compounds in rodents, for example, have as major targets epithelial cells of the skin, stomach, lung, and mammary gland but not the liver, the major site of AHH metabolism. While the relationship between toxic and carcinogenic responses may not be direct, it is likely that understanding the basis for modulation of the former will help predict the latter. The resistant hepatocyte model has proven useful in the analysis of chemically induced tumor formation in the rat liver (1), and a variant of this model involving resistance to terminal differentiation may be applicable to mouse epidermis (32). In this regard, elucidating the interaction of PAH with physiological effectors may be especially important.

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