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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and polycyclic aromatic hydrocarbons suppress retinoid-induced tissue transglutaminase in SCC-4 cultured human squamous carcinoma cells

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Retinoic acid and retinyl acetate induce tissue transglutaminase to high levels in cultured SCC-4 keratinocytes, increasing the enzyme specific activity over 50-fold under optimal conditions. Pretreatment of the cells for a day with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 3-methylcholanthrene (3-MC) or benzo[*a*]pyrene almost completely prevented the induction observed upon subsequent treatment with retinoic acid for 2 days. Similar aromatic compounds that do not induce aryl hydrocarbon hydroxylase (pyrene, dibenzofuran) did not exhibit this suppressive effect. The concentration dependence on TCDD for induction of aryl hydrocarbon hydroxylase was nearly identical to that for its suppression of transglutaminase induction, with half-maximal effects observed at ~20 pM in each instance. Similarly, the concentrations of 3-MC giving half-maximal stimulation of the hydroxylase and suppression of the transglutaminase were comparable (0.9 and 0.3 μM, respectively), although this agent was almost five orders of magnitude less potent than TCDD. These observations reveal a loss of cellular sensitivity to vitamin A mediated by the *Ah* receptor.

Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD*) is an extraordinarily potent tumor promoter (1,2) and teratogen (3) in model rodent bioassays. Since this compound is metabolized poorly, forming few DNA adducts (4), it presumably acts by altering cellular phenotypes through epigenetic mechanisms. TCDD induces the synthesis of a number of coordinately expressed enzyme activities, generally involved in xenobiotic metabolism (5). It binds with high affinity to the *Ah* receptor, which in turn binds to a DNA sequence element flanking the 5′ end of the cytochrome P-450 gene and thereby regulates its transcription (6). In addition, at least one other gene locus in mice (designated *hr*) governs the responsiveness of target tissues to the agent's chronic toxic action (7). Certain aromatic hydrocarbons also of current concern, such as polyhalogenated biphenyls and polycyclic aromatic hydrocarbons, bind to the *Ah* receptor and thus are believed to act at least in part by this mechanism. However, the latter compounds generally bind with much lower affinity to the receptor and, in addition, may be subject to rapid depletion by oxidative biotransformation, accounting for their relatively low potency in producing the chronic toxicity characteristic of TCDD and close structural analogs such as 2,3,7,8-tetrachlorodibenzofuran (5).

TCDD receptor-mediated events leading to tumor promotion or teratogenesis are poorly understood. Cellular alterations other than the induction of enzymes involved in xenobiotic metabolism are likely to be responsible, however (5). One possible way in which TCDD could seriously affect target cell function is indirect, by altering normal hormonal or physiological responses. An endocrine basis for TCDD action has been proposed in view of its interference in certain targets with glucocorticoid (8) and thyroid hormone (9,10) responses. In support of this concept, TCDD-treated rats exhibit reduced levels of hepatic and uterine estrogen receptor levels (11), prolactin receptor activity (12) and skeletal muscle glucocorticoid receptors (13). MCF-7 mammary carcinoma cells exhibit reduced estrogen responsiveness upon TCDD treatment (14). In cultured neoplastic human keratinocyte models, TCDD antagonizes the hydrocortisone-dependent differentiation displayed in SCC-13 cells (15) and significantly reduces the number of high affinity epidermal growth factor (EGF) receptors on the surface of SCC-12F cells (16). The latter action, also observed in cultured normal human epidermal cells, does not appear to involve thyroid hormone responses (17).

Vitamin A is essential for the maintenance of proper differentiated function of many epithelia and provides an obvious candidate with which TCDD might interfere. Vitamin A deficiency leads to squamous metaplasia at a variety of anatomic sites (18). Halogenated aromatic hydrocarbons are known to produce some effects resembling retinoid deficiency (19) including the squamous metaplasia of sebaceous ducts in the syndrome of chloracne. Since TCDD reduces hepatic storage but not circulating levels of vitamin A (20), whatever interference with retinoids it may manifest in target tissues presumably results from interactions within the cells. Recently we reported the presence of high levels of *tissue* transglutaminase, a soluble, retinoid-induced protein cross-linking enzyme in the human lingual squamous carcinoma line, SCC-4 (21). This enzyme has previously been shown to occur in cells of the myeloid lineage (22) and in mouse keratinocytes (23). Tissue transglutaminase is biochemically and immunochemically distinct from the particulate, retinoid-suppressed *keratinocyte* transglutaminase that is involved in cornified envelope production. While its function is currently unknown, it serves not only as a valuable indicator of retinoid action, but one that, as shown in the present experiments, is sensitive to the action of certain types of environmental toxicants. These experiments demonstrate the phenomenon of carcinogen-mediated altered target cell sensitivity to retinoids and offer the opportunity to explore its mechanistic underpinnings.

Materials and methods

SCC-4 cells, originally derived from a squamous carcinoma of the human tongue, were inoculated at a density of 10^5 cells/60 mm dish in the presence of a feeder layer of 5×10^5 lethally irradiated murine 3T3 cells (24). The medium consisted

*Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; 3-MC, 3-methylcholanthrene; EGF, epidermal growth factor; B[*a*]P, benzo[*a*]pyrene.

of three parts Dulbecco's modified Eagle's medium and one part Ham's F12 and was supplemented with 0.1 mM adenine, 5 μg/ml insulin, 5 μg/ml transferrin and 20 pM triiodothyronine. This medium was also supplemented with 2% fetal bovine serum (Hyclone, Logan, UT) during initial plating and 1% vitamin A-free, solvent-extracted fetal bovine serum (25) in subsequent medium changes. For transglutaminase assays, cultures were rinsed three times in medium, scraped onto 0.6 ml aliquots of cold 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM dithioerythritol, homogenized with 40 strokes in a glass Dounce homogenizer, and centrifuged at 100 000 *g*, 4°C, for 40 min. The resulting supernatant was assayed (25 μl aliquots containing 15–30 μg of protein) for incorporation of [^{3}H]-putrescine (New England Nuclear, Boston, MA) into reductively methylated casein (21). Aryl hydrocarbon hydroxylase was assayed in intact cultured cells by measuring the conversion of [^{3}H]benzo[*a*]pyrene (B[*a*]P) to polar metabolites (26). Cultures were rinsed four times in serum-free medium and exposed to 12 μM [^{3}H]B[*a*]P (New England Nuclear), 10 Ci/mol, for 90 min. The unmetabolized B[*a*]P was removed by hexane extraction and 0.5 ml of the alkaline aqueous phase containing the metabolites was neutralized with 2 ml of 1 M Tris (pH 7.7) and counted in 15 ml of Aquasol (27). Protein concentration was assayed (28) using Coomassie Brilliant Blue G-250 Reagent (Bio-Rad Laboratories, Richmond, CA). Results of the assays illustrated represent the mean and range of duplicate determinations. Retinoids, B[*a*]P, 3-methylcholanthrene (3-MC), phorbol-12-myristate-13-acetate, and pyrene were purchased from Sigma Chemical Co. (St. Louis, MO) and dibenzofuran from Ultra Scientific (Hope, RI). TCDD was a generous gift from Dr Howard Green.

Results

Although SCC-4 cells express little particulate transglutaminase (21), they are highly responsive to induction of the soluble tissue transglutaminase by retinoids. The concentration dependence of this stimulation is shown in Figure 1 for retinoic acid (panel A) and retinyl acetate (panel B), the acid and alcohol forms of vitamin A. Each agent produced substantial induction at 330 nM and >50-fold increase in specific activity at 3.3 μM, above which concentration toxic effects on growth rate and morphology were evident. In this type of experiment, 10 nM TCDD in the medium prevented the enzyme induction, as illustrated for retinyl acetate (panel B). The growth rate of SCC-4 cells is not altered by chronic exposure to TCDD even at 100 nM (29).

To study the structural specificity of this retinoid antagonism, the cultures were treated for 1 day as they reached confluence with one of several model compounds. Retinoic acid (3.3 μM) was then added and 2 days later the cells were harvested and assayed for transglutaminase activity. Under these conditions, as shown in Figure 2, retinoic acid treatment elicited a >8-fold stimulation of the enzyme, but TCDD prevented this increase above the unstimulated basal level. 3-MC and B[*a*]P (3.7 μM) were nearly as powerful as TCDD (10 nM), but the non-carcinogenic analogs pyrene and dibenzofuran (3.7 μM) were inactive. The potent epidermal tumor promoter phorbol-12-myristate-13-acetate (1 μM), an activator of protein kinase C, was also without effect.

This survey of compounds indicated that inducers of aryl hydrocarbon hydroxylase prevent the retinoid stimulation of tissue transglutaminase. To support the hypothesis that this phenomenon is mediated by the *Ah* receptor, the concentration dependences of both actions were compared. As shown in Figure 3, TCDD stimulated aryl hydrocarbon hydroxylase with an EC_{50} of ~20 pM and suppressed transglutaminase with a nearly identical IC_{50}. Similarly, the two actions of 3-MC were observed to occur with an EC_{50} and IC_{50} of ~0.3 and 0.9 μM, respectively. Thus, the cells were 4–5 orders of magnitude more sensitive to TCDD than to the polycyclic compound, similar to the *Ah*-mediated induction of a keratinization-like response in mouse teratoma-derived XB keratinocytes (30). 3-MC induced a relatively low level of aryl hydrocarbon hydroxylase activity compared to TCDD under the conditions employed. This contrast was due at least partially to depletion of the polycyclic compound

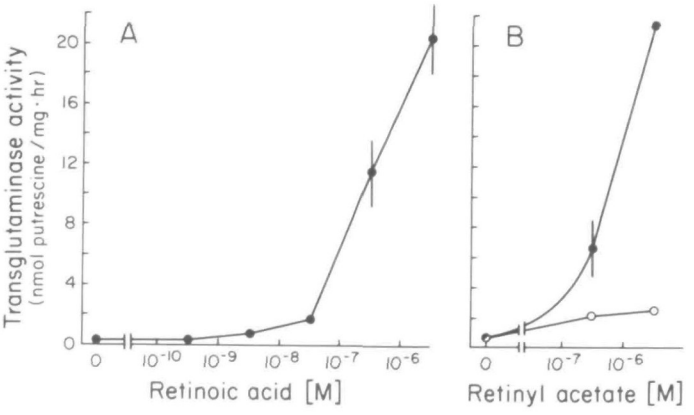

Fig. 1. Effect of 3.3 μM retinoic acid (**A**) and 3.3 μM retinyl acetate (**B**) without (closed circles) or with (open circles) 10 nM TCDD on tissue transglutaminase activity in SCC-4 cells. Assays were performed on cells exposed during log phase growth and maintained under these conditions until 1 week after confluence.

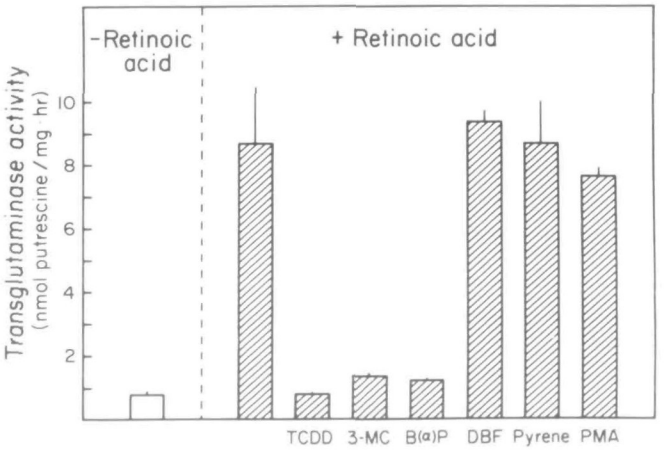

Fig. 2. Specificity of the inhibition of retinoic acid-stimulated tissue transglutaminase activity. Upon reaching confluence, the cultures were exposed for 24 h to the test compounds indicated, at which time retinoic acid (3.3 μM) was added for 48 h prior to assay. Values represent the mean and range of duplicate determinations. All test compounds were 3.7 μM except TCDD which was 10 nM and PMA which was 1 μM. 3-MC: 3-methylcholanthrene; B[*a*]P: benzo[*a*]pyrene; DBF: dibenzofuran; PMA: phorbol 12-myristate 13-acetate.

by metabolism. Variation of the experimental conditions involving shorter exposure periods to 3-MC prior to aryl hydrocarbon hydroxylase assay (1 day instead of 3 days, for example) gave increased induction, commonly 5-fold (data not shown). These alterations had no detectable effect on the degree of transglutaminase suppression.

Discussion

In the present experiments, the efficacies of the polycyclic aromatic hydrocarbon compounds are compatible with mediation of their action through the *Ah* receptor. The considerably higher concentrations required than for TCDD reflect the known lower affinities of the polycyclics for the receptor and their effective removal by the induced aryl hydrocarbon hydroxylase. TCDD (1,2,31) and carcinogenic poycyclic aromatic hydrocarbons have tumor-promoting activity which is usually described as inappropriate non-genotoxic growth stimulation of initiated cells (32). If, as is generally accepted, this activity of TCDD

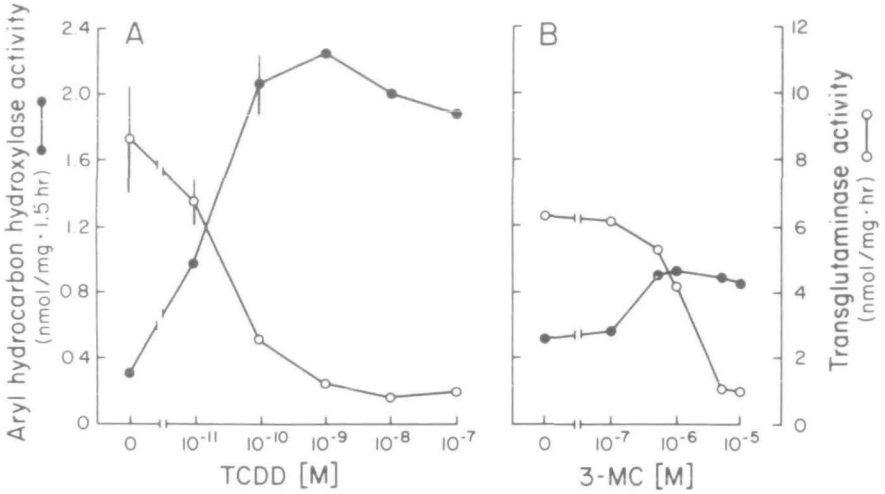

Fig. 3. Concentration dependence of the suppression of retinoic acid-stimulated tissue transglutaminase activity (open circles) and induction of AHH activity (closed circles) by TCDD (**A**) or 3-MC (**B**). Cells were treated as in Figure 2.

is receptor-mediated, then at least some of the tumor-promoting ability of the polycyclics presumably is conferred in the same way, independent of the generation of DNA-damaging electrophiles. The present demonstration of a loss of retinoid sensitivity is not obviously related to growth regulation but is plausibly symptomatic of a more global response to these promoters. Similarly, TCDD has been reported to reduce adrenocorticotropin-stimulated steroidogenesis in bovine adrenocortical cells by disturbing the intracellular cholesterol distribution (33). Recent evidence points to a role for common cellular events in enhancing the incidence or detection of malignant progression of cell populations treated with carcinogens, including radiation and polycyclic aromatic hydrocarbons (34,35). The obscure nature of this phenomenon emphasizes the desirability of investigating the common changes such agents induce in treated cells and the mechanisms by which these changes are produced. The inactivity of phorbol myristate acetate in this system emphasizes the potentially varied nature of changes that can be produced in target cells during non-genotoxic promotion.

One of the perplexing features of TCDD action is its extreme species and tissue dependence in producing pathological change in target cells, including those of epidermis and its appendages (5). Loss of sensitivity of certain cells to vitamin A (or the underlying disorganization which this represents) could well lead to marked changes in phenotype. Indeed, determining the degree to which a spectrum of retinoid responses are altered in a given target cell type would be valuable. The possible role of induced enzymatic metabolism in inactivation of retinoids within the treated cells then may merit investigation. However, while such inactivation could contribute to TCDD induction of squamous metaplasia in some target sites (e.g. ducts of sebaceous glands), little keratinocyte reprogramming is apparent in other targets.

In a number of instances, as in the present case, TCDD effects in target cells include a loss of responsiveness to specific effectors (hormones, vitamin A). TCDD-mediated intracellular metabolic inactivation of such effectors would provide a plausible explanation of such action, but evidence in support of this general scenario is weak. For example, enhanced inactivation of hydrocortisone in SCC-13 cells could not be detected despite TCDD suppression of the glucocorticoid action (15). Retinoid metabolism in SCC-4 cells has not been examined. However, in the malignant keratinocyte line SCC-12B2, derived from a human epidermal squamous cell carcinoma (24), TCDD may potentiate the action of retinyl acetate by antagonizing its suppression by hydrocortisone (36; A.L.Rubin, W.O'Callahan, X.Rong and R.H.Rice, unpublished data). In any event, further analysis of the actual state of differentiation in such afflicted cells may be especially valuable in understanding their responses to TCDD and consequent pathological behavior.

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