ANTAGONISTIC EFFECTS OF RETINOIC ACID ON THE 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN INDUCED DIFFERENTIATION OF *IN VITRO* CULTURED HUMAN KERATINOCYTES.

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INTRODUCTION

Polychlorinated aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are among the most toxic compounds produced by man[1] with also strong carcinogenic properties[2]. Because of their highly stable nature and lipophylicity, they have become widespread in the environment[1,2]. One of the most eminent responses of man to TCDD intoxication is development of chloracne: a disturbance of the normal differentiation process of keratinocytes[3]. TCDD-induced differentiation can also be observed in human keratinocytes in primary culture using different biochemical and morphological parameters as differentiation markers[4,5].

Retinoids are very potent compounds in regulating the epidermal keratinocyte proliferation and differentiation both *in vivo* and *in vitro*[6,7]. Studies in different species show an altered retinoid metabolism after TCDD-intoxication[8,9]. Many effects of dioxin resemble the clinical status that can be noticed after vitamin A-deficiency. In this study we have investigated the interaction of dioxin and retinoic acid with respect to their effects on the differentiation of human keratinocytes in primary culture.

EXPERIMENTAL

Cell Culture. Normal human keratinocytes, derived from both neonatal or adult foreskin explants after compliance with ethical regulations, were isolated and seeded without feeder cells into collagen-precoated 6-well multidishes (35-mm-diameter wells, 2-5x10^5 cells/well) basically according to Rheinwald[10]. The cells were cultured in either Dulbecco's Modified Eagle's Medium /Ham's F-12 medium (3:1 v/v) supplemented with

EGF, hydrocortisone, cholera toxin and 5% fetal calf serum, or in serum-free KGM medium (Clonetics).

Treatment. Aliquots of concentrated stock solutions of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and retinoic acid were prepared in DMSO. These stocks were diluted in culture medium prior to addition to cells. Treatment of the cells with TCDD and/or retinoic acid was achieved by adding 20 µl of the diluted stocks to the ^{35}S-methionine-containing medium. The concentration of DMSO in medium never exceeded 0.2%(v/v).

Quantification of the Cross Linked Evelopes (CLEs). As a marker for keratinocyte differentiation we quantified the amount of ^{35}S-methionine-labelled proteins incorporated into the cross linked envelopes (CLEs) essentially according to the method of King *et al.*[11]. In short, after reaching confluency the medium was removed and cells were labeled with 1µCi/ml ^{35}S-Methionine for at least 6 h, followed by a four day simultaneous treatment with the bioactive substances. The cells were washed twice with PBS. In order to prevent the CLEs from clotting by uncoiled DNA after disruption of the cells by SDS, we permeabilized the cells with NP40 and fragmented the DNA in the intact nucleus by a 30 min treatment with DNase I at 37°C according to the method of Stewart *et al.*[12]. Cystamine (5 mM) was added to inhibit transglutaminase activity. CLEs were isolated by adding 2.5% SDS and 50 mM DTT and collected on GF/B filters. The ratio of the amount of ^{35}S-methionine incorporated in CLEs and the amount of ^{35}S-methionine incorporated in the Total Amount (TA) of intact cells (CLE/TA) was considered to be a quantitative parameter for terminal keratinocyte differentiation.

RESULTS AND DISCUSSION

Treatment of the keratinocytes with increasing concentrations of TCDD (10^{-13} to 10^{-8} M) showed a 5 to 10-fold increase of CLE-formation as quantified by ^{35}S-methionine incorporation (Fig.1; open circles). Essentially the same results can be obtained by counting the number of CLEs resulting in an increase of CLEs from 15% in controls to 75% at a TCDD-concentration of 10^{-8} M (data not shown). During the 4-day incubation period with TCDD the increase in the number of differentiating cells is accompanied by a decrease of the number of proliferating cells with approximately 50% (data not shown). Treatment of the cells with increasing concentrations of retinoic acid (10^{-10} to 10^{-6} M retinoic acid) showed a slight decrease in CLE formation (Fig.1; open squares). However, simultaneous treatment with both 10^{-8} M TCDD and increasing concentrations of retinoic acid resulted in a marked decrease in TCDD-induced differentiation (Fig.1; closed squares), reaching control levels at 10^{-5} M retinoic acid (results not shown). In addition, the TCDD-induced differentiation of keratinocytes can be antagonized dramatically by simultaneous incubation with 10^{-6} retinoic acid (Fig.1; closed circles). These results were obtained in different individuals, however differences in both TCDD and retinoic acid responses could be observed, demonstrating an individual human variance.

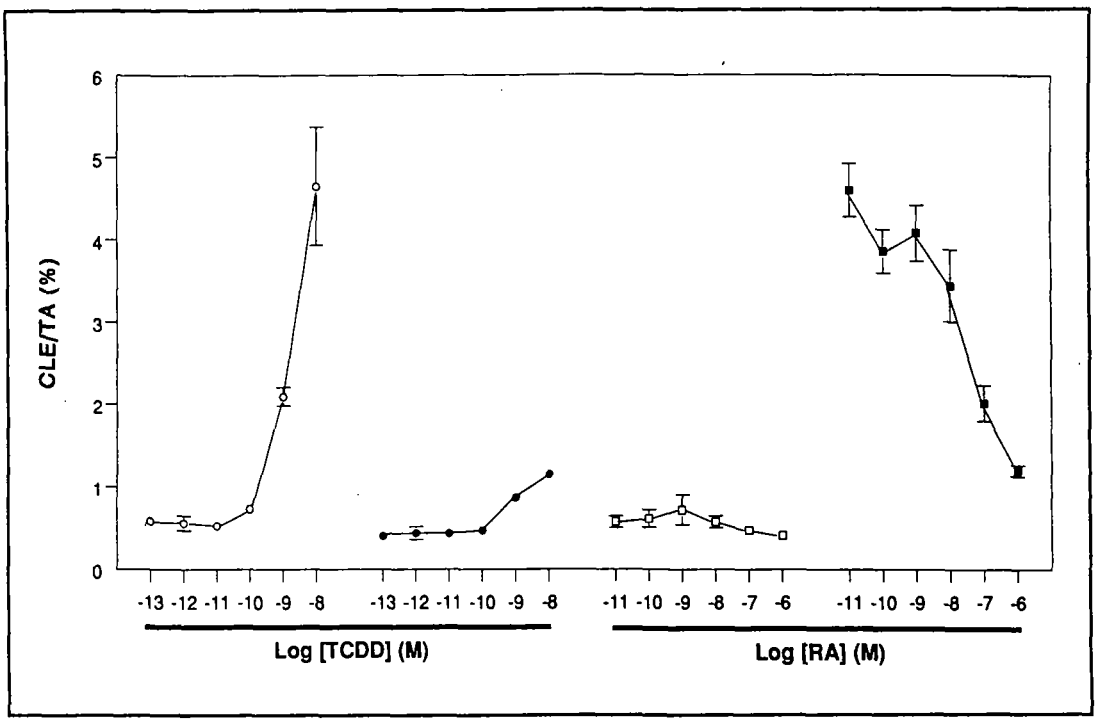

Figure 1. The effects of TCDD and retinoic acid (RA) on the keratinocyte differentiation quantified by the amount of ^{35}S-methionine incorporated in CLEs: CLE/TA (%). Open circles; TCDD-concentration range without retinoic acid, closed circles; with 10^{-6} retinoic acid, open squares; retinoic acid-concentration range without TCDD, closed squares; with 10^{-8} TCDD.

Competition studies with TCDD and retinoic acid indicate that retinoic acid is a non-competitive antagonist of the TCDD-induced differentiation.

Many responses of cells are altered by culture conditions or factors in media. Therefore, we repeated the experiment with cells cultured in defined serum-free KGM medium (Clonetics). The same antagonistic properties of retinoic acid could be observed, however the quantity of the effect was diminished due to the proliferative properties of the medium. In addition, elimination of hydrocortisone increased the TCDD-induced differentiation without affecting the antagonistic effects of retinoic acid. In some cell types the human thyroid hormone receptor forms a heterodimer with the human retinoic acid receptor and both receptors contribute to the regulation of gene transcription[13]. Therefore, we investigated the effect of triiodothyronine (T_3) with concentrations ranging from 10^{-10} to 10^{-6} M on the TCDD-induced differentiation (data not shown). No antagonistic effects could be observed, suggesting a specific antagonistic effect of retinoic acid on the dioxin-induced keratinocyte differentiation.

CONCLUSIONS

We conclude that retinoids are potent and specific inhibitors of dioxin-induced differentiation. The presented results indicate a possible therapeutic role of retinoic acid as an antagonist for dioxin-induced skin abnormalities.

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