Neoplastic Transformation of Immortalized Human Keratinocytes by 2,3,7,8-
Tetrachlorodibenzo-p-dioxin

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Abstract

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the most powerful carcinogen ever tested in animals. Recent epidemiological studies have suggested its carcinogenic potential in humans. In the present study, nontumorigenic human epidermal keratinocytes immortalized by adenovirus 12-simian virus 40 (Ad12-SV40) were transformed by exposures of TCDD equal to or greater than 0.1 nM for 2 wk. These transformed cells showed morphological alterations and induced carcinomas when transplanted into nude mice, whereas no such transformation phenotypes were observed with exposures of less than 0.1 nM for 2 wk. Primary human epithelial keratinocytes exposed to various concentrations of TCDD failed to show any evidence of transformation. Induction of aryl hydrocarbon hydroxylase activity was dose dependent, as was transformation. Thus, the carcinogenicity of TCDD in this human cell system appears to be an Ah receptor-mediated process. The present study represents the first evidence of neoplastic conversion of human cells exposed to this environmentally important chemical.

Introduction

TCDD,2 one of the most toxic man-made compounds, bioaccumulates in animals and humans and is widely dispersed in the environment (1). Despite extensive research during the past two decades, the mechanisms of TCDD-induced carcinogenesis are poorly understood, and its carcinogenic potential in humans is not clear. Exposure to TCDD has been shown to be teratogenic (2) and carcinogenic (3) in various animal species. In addition, this compound is known to be an extraordinarily potent tumor promoter in rodent bioassays (4). Since TCDD forms few DNA adducts and is negative in multigenicity assays (5, 6), this compound appears to be nongenotoxic and to act by epigenetic mechanisms. While in humans, only chloracne and hyperkeratosis (7) have been unequivocally attributed to TCDD exposure, many other biological effects on humans including cancer are highly suspected (8). Recently, epidemiological studies have suggested that TCDD is a human carcinogen at least at high doses (9). Although TCDD is a powerful carcinogen in several species (10), limited model systems exist to study carcinogenicity of this compound at the cellular level. TCDD acts as a promoter on mouse embryonic fibroblasts C3H10T/1/2 (11) and rat tracheal epithelial cells (12) initiated with MNNG. So far, there has been no report of its carcinogenic potential in human cells in culture. The present study was designed to characterize the carcinogenic potential of TCDD in human cells exposed to TCDD. We used a nontumorigenic, immortalized human epithelial keratinocyte cell line (RHEK-1) (13).

RHEK-1 cells can be malignantly converted to tumorigenic cells by chemical carcinogens, X-ray irradiation, oncogenic viruses, and an activated H-ras oncogene (14–16). This cell line has thus proven useful in studying multistep human foreskin epithelial cell carcinogenesis. Since TCDD effects are target organ and species specific and since one of the sensitive target organs in humans is skin (17), use of this particular cell system offers an advantage in evaluating human toxicity to TCDD.

Materials and Methods

Cell Cultures and Media. The human epidermal keratinocyte line, designated RHEK-1, was used at passage 31 for these transformation studies. This cell line was established from primary foreskin epidermal keratinocytes after infection with the Ad12-SV40 hybrid virus (13). These cells did not produce virus, had a "flat" epithelial morphology, and expressed a number of markers associated with epithelial cells. This cell line also contained the SV40 tumor antigen but was not tumorigenic in nude mice. Growth and maintenance medium for these cells consisted of Dulbecco's modified minimal essential medium with 10% fetal bovine serum, hydrocortisone (5 μg/ml), penicillin G (50 units/ml), and streptomycin (50 μg/ml). Primary epithelial keratinocytes were grown in keratinocyte growth medium (Clonetics, San Diego, CA).

Chemicals. TCDD was obtained from KOR Biochemical (Cambridge, MA). The purity of this compound was >99% as assayed by analytical high-pressure liquid chromatography. TCDD was dissolved in DMSO, and aliquots (100 μl) were stored at −70°C. Various concentrations of TCDD were prepared by direct dilution of 100 μl aliquots into appropriate media. All media including control contained a final concentration of 0.1% DMSO.

Transformation Assay. One day after primary human epidermal keratinocytes or the RHEK-1 line was plated at 104 cells per T-75 (75 cm2) Falcon plastic flask, the medium was removed and replaced with medium containing the various ranges of TCDD in DMSO (<0.1%). The control medium contained 0.1% DMSO. After 2 wk of exposure, the cultures were washed, fed again with TCDD-free growth medium, and passaged by trypsin treatment every 10 to 14 days. Cultures were observed biweekly for changes in morphology and growth pattern.

Colonial Formation in Soft Agar. Cell suspension (1 × 105 cells per ml) in 5 ml of 0.35% Noble agar was overlaid in a 60-mm dish containing a 0.6% agar base. Viable colonies were scored at 21 days.

Tumorigenicity in Nude Mice. Adult NIH/Swiss athymic nude mice were inoculated s.c. with 1 × 106 and freshly trypsin-treated cells to determine tumorigenicity.

AHY Assay. Induction of AHY activity by TCDD in RHEK-1 cells was assayed by the method of Jansing and Shain (18). Briefly, cells were washed twice with cold PBS, harvested by scraping with a rubber policeman, and centrifuged at 1000 rpm for 10 min at 4°C. The pellets were resuspended in 0.5 ml of buffer containing 25 mM Tris-HCl (pH 7.4)-0.25 mM sucrose and then sonicated 3 times for 10 s in an ice bath. The lysate (1 mg of protein) was assayed for AHY activity.

Results

Transformation of RHEK-1 Cells by TCDD. When primary human epithelial cells were exposed to various concentrations

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2The abbreviations used are: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; DMSO, dimethyl sulfoxide; AHY, aryl hydrocarbon hydroxylase.
of TCDD, no evidence of transformation phenotypes was observed in the treated and untreated cultures. Neither untreated nor treated cultures were able to grow beyond two or three subcultures. The cells progressively deteriorated and died (data not shown). While RHEK-1 cells exposed to TCDD at 0.1 nM or above for 2 wk showed apparent morphological changes of cells and an abnormal pattern of growth after fifth to sixth subcultures, 70 to 84 days after treatment, no such changes were observed at doses lower than 0.1 nM. In addition, a 3-day exposure of TCDD on this cell line did not show any significant alterations in any dose groups after the sixth subcultures (data not shown).

As reported previously for a variety of other cell cultures (19), the growth rates of RHEK-1 cells were not affected by TCDD, indicating that phenotypic changes of these cells are not the result of excess cytotoxicity. The morphological changes observed at doses equal to or higher than 0.1 nM were similar to those observed with other types of carcinogens in this cell system; namely (16), the transformed cells began to pile up in focal areas, form small projections, and release round cells from the foci (Fig. 1, e to f). In contrast, the cellular morphology remained unchanged at doses lower than 0.1 nM and continued to grow as nonoverlapping round-to-polygonal adherent cells that were flat and cobblestone-like in appearance (Fig. 1, a and b).

Characteristics of TCDD-transformed RHEK-1 Cell Lines. The TCDD-transformed cells were further characterized by quantitative differences in growth properties, such as saturation density and soft agar colony-forming efficiency associated with the neoplastic phenotypes. The saturation density of transformed cells (≥0.1 nM) was approximately 1.5 to 2 times higher than untransformed cultures (<0.1 nM) (Table 1). In addition, the chemical transformants grew in soft agar with colony-forming efficiencies of 0.04 to 0.2%, whereas the untransformed cells (<0.1 nM) did not grow in soft agar (Table 1).

Evidence of the human origin of all the cell lines was obtained by isoenzyme analysis and cell membrane species-specific immunofluorescence. The relatedness of the transformed cells to the parental RHEK-1 cells was further established by chromosomal analysis. All the cell lines are aneuploid human male (XY) derivatives with chromosome counts in the near-diploid range. Both transformed and untransformed cells had similar marker chromosomes.

When athymic nude mice were inoculated s.c. with 10⁷ TCDD-transformed cells, the animals developed tumors within 8 wk. Such tumors were diagnosed as squamous cell carcinomas, and cells were typically arranged in sheets, with individual cells often containing keratohyalin granules or prekeratin (Fig. 1g). Cultures established from these tumors were similar to TCDD-transformed cells, were confirmed as human and resembled the cells of origin by karyological analysis (Fig. 1A). In contrast, s.c. injection of 10⁷ untransformed cells (<0.1 nM) into nude mice produced regressing cystic nodules containing epidermal cells.

Since RHEK-1 cells can be transformed by an activated H-ras oncogene transfection and become tumorigenic (16), TCDD-transformed and H-ras oncogene-transformed RHEK-1 cells were analyzed by immunoprecipitation to determine if TCDD-induced transformation is associated with the ras oncogene product, p21. In contrast to the findings in H-ras gene-transformed cells, neither altered mobility nor increased expression of p21 was observed in TCDD-transformed cells (data not shown). Moreover, transfection of NIH 3T3 cells with genomic DNA derived from TCDD-transformed cultures failed to show detectable foci. These findings suggest that the activation of the ras oncogene may not be involved in the TCDD-induced transformation of RHEK-1 cells.

Induction of AHH Activity. It has been reported that the toxic effects of TCDD and its congeners are highly correlated with the induction of cytochrome P-450s (1). Thus, induction of these particular enzymes has been suggested as a surrogate to measure sensitive responses to dioxins in all species. In the present study, concentration-dependent induction of AHH activity was observed by TCDD (Fig. 2). In addition, exposure of RHEK-1 cells to TCDD also induced a dose-dependent increase of ethoxyresorufin-O-deethylase activity, which was similar to that of AH activity (data not shown). While maximum induction of AHH activity occurred at a dose of 30 nM, the highest efficiency of soft agar colony formation of transformed cells was observed at a dose of 1.0 nM. The different concentrations of TCDD involved in the induction of AHH activity and transformation indicate that these two responses may occur by different sets of dioxin-response genes which may respond to a different concentration of TCDD.

Discussion

The present study represents the first reported malignant transformation of human cells by Ad12-SV40 virus and TCDD. At least two and possibly more alterations in cell growth properties seem to be required. A measurable event was the acquisition of apparently unlimited growth potential as a result of Ad12-SV40 infection. Treatment of nontumorigenic early passage Ad12-SV40 immortalized epithelial cells with TCDD resulted in further changes in their growth properties. Morphological alterations as well as the ability to grow in soft agar and to form rapidly growing squamous cell carcinomas in athymic nude mice appeared to be concomitantly acquired properties of the TCDD-transformed cells. The significance of the combined effects of Ad12-SV40 virus and TCDD on the induction of neoplastic human epithelial cells is emphasized by the inability of TCDD alone to produce continued proliferation of primary epithelial cells under our assay conditions. This also indicates that TCDD is inactive as an initiator or complete carcinogen in primary human epithelial cells. As reported in other in vivo and in vitro studies (4, 11), the role of TCDD in this cell system appears to be as a tumor promoter associated with the viral-chemical interaction (20). Unlike the rapid transformation of RHEK-1 cells after Kirsten murine sarcoma virus infection (13), growth alterations associated with TCDD treatment were delayed in their appearance and required several subcultures for visualization. These findings suggest that multiple cell divisions are required for fixation and expression of the transformed phenotype in response to TCDD. It is possible that more than one genetic lesion may be required as well. Cooperating cellular or viral oncogenes have also been shown to induce malignant transformation of embryonic rodent fibroblasts (21, 22). In addition, the combined action of tumor virus and chemical carcinogens has been demonstrated to produce neoplastic transformation of human epithelial cells and rodent fibroblasts (14, 23). Our ability to obtain malignant transformation as a result of TCDD treatment of Ad12-SV40-altered human epithelial cells provides additional support for a multistep process of neoplastic conversion.

The conversion of nontumorigenic RHEK-1 cells by TCDD to a tumorigenic phenotype suggests that cellular oncogenes are involved in this process.
NEOPLASTIC TRANSFORMATION OF HUMAN KERATINOCYTES BY TCDD

Fig. 1. Human epidermal keratinocytes (RHEK-1) treated with TCDD for 2 wk, followed by 7 subcultures in nutrient medium. Control (0.1% DMSO) (a), 0.03 nM (b), 0.1 nM (c), 0.3 nM (d), 1.0 nM (e), 3.0 nM (f), in vivo tumor induced by RHEK-1 cells treated with 0.3 nM, moderately well-differentiated squamous cell carcinoma (g), and typical field of a culture originated from a primary tumor induced by RHEK-1 cells treated with 0.3 nM (h).
may be activated as part of the process. Recent studies indicated that the effects of TCDD treatment, both in vivo and in vitro, in certain species are implicated with c-ras, c-erb-A, and c-src gene expression (24, 25). However, our results suggest that the ras oncogene, which has been commonly implicated in chemical carcinogen-induced animal tumors and spontaneous human tumors (26), was not activated in the transformants so far analyzed.

Since TCDD affects proliferation and differentiation of human keratinocytes and since some of its effects are known to be associated with various growth factors (27–29), the tumorigenic response of RHEK-1 cells to TCDD may be modulated via unknown epigenetic mechanisms. The roles of these epigenetic effects on the expression of transformed phenotypes warrant further investigation in this cell system.

The induction of transformation and AHH activity in higher dose groups indicates the possibility that this cell line has the Ah receptor and that the transformational events may be a receptor-mediated process. While a 3-day exposure of TCDD did not show any significant transformation (data not shown), a 2-wk exposure showed dose-dependent transformations. These findings indicate that TCDD acts on this cell system in a time- and dose-dependent manner.

Considering that interspecies differences in response to TCDD are an especially important factor in evaluating human risks from experimental animals, the use of human epithelial cells, of which the cell type TCDD-induced toxicity is most prominent (1) and from which most cancer origins are derived (13), may add further significance to the present findings of risk assessment. While additional studies are required to understand to what extent events in cell cultures could be extrapolated to an in vivo system and be used for risk assessment, this in vitro model provides a valuable tool to study mechanisms of TCDD-induced carcinogenesis in human cells and to screen the potential carcinogenicity of dioxin-related compounds in human cells.

References


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