Chemical Transformation of Human Keratinocytes by 2,3,7,8-Tetrachlorodibenxo-ρ-dioxin

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ABSTACT : 2,3,7,8-Tetrachlorodibenzo-ρ-dioxin (TCDD) is a ubiquitous, persistent environmental contaminant and the most powerful carcinogen categorized by IARC. Although the mechanism of carcinogenesis by TCDD is poorly understood, several studies have shown that the skin is one of target organs for TCDD. In this study, we investigated the neoplastic transformation of human keratinocyte-derived cell line, HaCaT, by chemical transformation method using *N*-methyl-*N'*-nitro-*N*-nitorsoguanidine (MNNG) and TCDD. We found that subsequent exposure to TCDD for 3 weeks after initial exposure to MNNG markedly induced transformed cells. It was suggested that TCDD can act as a potent promoter in HaCaT cells. Furthermore, these transformed cells showed morphological alternations in soft agar and increased telomerase activity. Therefore, the TCDD treatment of HaCaT cells by initiated with MNNG could promote neoplastic transformation without stimulation by exogenous growth factors. As a result, TCDD had a strong potency as a promoter in nontumorigenic immortalized human epidermal keratinocytes.

Key words : **2,3,7,8-tetrachlorodibenzo-ρ-dioxin, *N*-methyl-*N'*-nitro-*N*-nitorsoguanidine, HaCaT, transformation, soft agar, telomerase**

Introduction

2,3,7,8-Tetrachlorodibenzo-ρ-dioxin (TCDD) is a ubiquitous environmental contaminant and the prototype for a class of halogenated aromatic hydrocarbons (HAHs) known as dioxins (Safe, 1990; Van den Berg *et al.*, 1998). TCDD is due to its environmental and biological persistence, which may result in bioconcentration and bioaccumulation while moving up the food chain (Schecter *et al.*, 2001). In a recent re-evaluation of the carcinogenicity of TCDD, TCDD has been classified as a 'class I' human carcinogen by the International Agency for Research on Cancer (IARC) (IARC, 1997). TCDD is not a mutagen and does not interact with DNA (Poland and Glover, 1979; Geiger and Neal, 1981). There is growing evidence indicating that TCDD is a potent tumor promoter in rat and mouse livers, as well as in mouse skin (Herbert *et al.*, 1990; Poland and Kuntson, 1982; Tanaka *et al.*, 1989).

TCDD is associated with several types of human cancers and human skin is one of target organs for TCDD (Bertazzi *et al.*, 1992; Johnson, 1993). Generally, the mouse skin carcinogenesis model has been used to assess whether or not the chemical such as TCDD carrying a carcinogenic hazard to humans. However, because human cells showed a resistance to transformation by TCDD (Tayler, 1979), traditional examination of TCDD-induced carcinogenesis in mouse skin has been limited to evaluate tumor-promoting potency of TCDD on human skin.

Loertscher *et al.* (2001a) introduced an organotypic culture system for examination of the effects of TCDD on human skin. In addition, cultured immortalized human keratinocyte cell lines have been used as the models to understand how TCDD perturbs keratinocyte function (Rhim *et al.*, 1985; Yang *et al.*, 1992). Despite increased the evidence to show carcinogenicity by TCDD on human skin systems, it is difficult to link these morphological alternations of normal human keratinocytes to TCDD *in vitro*. Furthermore, it may not provide the responsible mechanisms of clonal expansion in TCDD-induced carcinogenesis.

TCDD has been shown to induce an inhibition of proliferation, subsequently eliciting apoptotic cell death

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in a variety of cell types (Heimler *et al.*, 1998; Hossain *et al.*, 1998). However, several studies indicated that TCDD had a tumorigenic effect in human cells that involved in the dysregulation of cellular proliferation through alternation of growth regulatory genes (Conner *et al.*, 1994; Greenlee *et al.*, 1987; Yang *et al.*, 1999) and through suppression of apoptosis (Loerscher *et al.*, 2001b). Recently, Ray and Swanson (2004) reported that TCDD induced the immortalization of normal human ketatinocytes through an aryl hydrocarbon receptor (AhR)-mediated bypass of cellular senescence. It was indicated that inappropriate alternation of the cellular growth program by TCDD was an important mechanism on TCDD-induced carcinogenesis.

It has been reported that telomerase was upregulated during mouse tumorigenesis (Blasco *et al.*, 1997; Broccoli *et al.*, 1996). Several studies suggested that the increase of telomerase activity resulted in immortal growth of cultured human cells (Bodnar *et al.*, 1998; Kiyono *et al.*, 1998; Morales *et al.*, 1999). Furthermore, telomerase activation was able to transform the cultured primary human cells into neoplastic cells (Hahn *et al.*, 1999). Although a great deal is known about telomerase-based tumorigenesis in human cells, much less is known about the mechanisms by which TCDD causes neoplastic transformation.

To elucidate the biological effect of TCDD as a potent promoter on human keratinocytes, we investigated the neoplastic transformation with the method of chemical transformation in human keratinocyte-derived HaCaT cells using *N*-methyl-*N'*-nitro-*N*-nitorsoguanidine (MNNG) and TCDD. In addition, we report that TCDD immortalized primary keratinocytes through an alternation of cellular growth that involves the increase of telomerase activity.

Materials and Methods

Chemicals

2,3,7,8-Tetrachlorodibenzo-ρ-dioxin (TCDD) was purchased from the Cambridge Isotope Laboratories, Inc (USA). *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), acetone, dimethyl sulfoxide (DMSO) and sodium bicarbonate were obtained from Sigma Chemical Co. (USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA, penicillinstreptomycin, and sodium pyruvate solution were purchased from Gibco BRL (USA).

Cell culture and media

The human epidermal keratinocyte cell line, designated HaCaT was provided by Dr. In-Soo Lee (KFDA, Seoul, Korea). Human normal skin fibroblast Detroit 551 (passage 15) and epidermal carcinoma cell line A431 (passage 8) were obtained from Korean Cell Line Bank (Seoul, Korea). HaCaT, Detroit551 and A431 cells were cultured in Dulbecco modified Eagle medium containing 10% FBS and 1% penicillin-streptomycin at 37°C in a 5% CO_2 and 95% humidity. All of our experiments were performed on cultures that were at 70% confluence, when cells still in log phase growth.

Chemical transformation

For the formation of transformed cell lines in HaCaT, a recently published protocol by Fang *et al.* (2001) was modified used. Briefly, cells were seeded at 1.0×10^5 cells/flask (25 cm^2) in 5 ml DMEM supplemented with 10% FBS. One day after HaCaT cells were plated at the flask, the cells were treated with 0.1 μg/ml MNNG for 24 hours, then medium was removed and replaced with the medium containing 100 nM TCDD in DMSO (< 0.1%) for 20 days with two medium changes per week. After 20 days of exposure to TCDD, several foci that showed dense multilayering of cells among transformed foci were subcultured for three passages in TCDD-free growth media, and developmented for TCDD-transformed HaCaT cells. Four groups were designed, including untreated control (not treated and not participating in the *in vitro* chemical transformation process, treated only with 0.1% DMSO), MNNG (treated only 0.1 μg/ml MNNG), TCDD (treated only 100 nM TCDD), and TCDD-transformed (three-passaged transformed cells) groups (Fig. 1).

Colony formation in soft agar

In order to investigate the growth properties of TCDD-transformed cells, the cells were cultured with low cell density in soft agar analysis system for 4 weeks. Untreated control and TCDD-transformed cell suspension (5×10^3 cells per well) in 3 ml of 0.30% Noble agar were overlaid in 6 well plates containing a 0.5% Noble agar. Human epidermal carcinoma cell line, A431 cells were used as positive control and human normal skin fibroblast Detroit551 cells were used as negative control. After solidification at room temperature for 2 hours, plates were incubated in a 5% CO_2 incubator at 37°C. Colonies were formed after 4 weeks

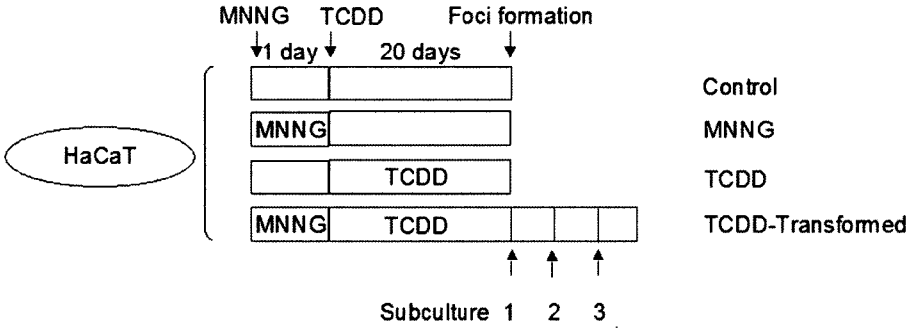

Fig. 1. Chemical transformation of HaCaT cells by MNNG and TCDD. The HaCaT cells were treated with 0.1 μg/ml MNNG for 24 hours until the cells reached subconfluence, then were treated with 100 nM TCDD for 20 days. After 20 days, cells were transformed. Several foci among transformed foci were subcultured for four passages. Four groups were designed, including control (untreated HaCaT cells, only treated with 0.01% DMSO), MNNG (only treated with MNNG), TCDD (only treated with TCDD), and TCDD-transformed (three-passage transformed cells) groups.

incubation. Colonies of cells were stained by adding 0.5 ml of 0.005% crystalviolet in PBS to each well and incubated for overnight at 37°C. And then photographed using a Nikon X-370 camera mounted on a Zeiss Axiovert 10 microscope at 200 × magnification.

Telomerase Assay of TCDD-transformed HaCaT cells

Telomerase activity was measured by telomeric repeats amplification protocol (TRAP) assay with $TRAP_{EZE}$ Telomerase Detection Kit (Oncor, Geithersberg, USA). Each trypsinized cells (1×10^5) were lysed in 0.2 ml of ice-cold buffer containing 0.05% CHAPS (3-[3-chloamidopropyl]-dimethylammonio-1-propane sulfonate), 10 mM Tris-Cl, 1 mM $MgCl_2$, 1 mM EGTA, 5 mM β-mercaptoethanol, 0.1 mM 4-(2-aminoethyl)-bensene-sulfonyl fluoride HCl and 10% glycerol. Samples were homogenized by repeated pipetting, hold on ice for 30 min and centrifuged at 16,000 g for 20 min at 4°C. The protein concentration of the supernatant was measured by a Bradford assay (Bio-Rad Laboratories, Hercules, CA). The cell extract was diluted to the concentration of 2.5 μg/μl of protein, flash frozen in liquid nitrogen and stored at −80°C until use. The TRAP assay was performed in two steps (Jang *et al.*, 2001). 5 μg protein of the extract was incubated at 30°C for 30 min in 50 μl of reaction solution containing 20 mM Tris-Cl, 63 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 0.05% glycerol, 25 mM dNTP mix, 0.1 μg biotin-labeled TS primer (5'-AATCCGTCGAGCAGAG TT-3') and 0.1 μg reverse primer and heated to 94°C for 3 min. And then followed by 30 cycles (94°C for 30 sec and 52°C for 30 sec) of polymerase chain reaction (PCR) amplification using 2 units of Taq DNA polymerase with GeneAmp Thermal Cycler Model 2400 (Perkin-Elmer, Foster, USA). The PCR products were analyzed by electrophoresis on a 10% non-denaturing polyacrylamide gels and stained with Etidiumbromide. Telomeric adders were analyzed using GelDac image software (RioRad Laboratories, Hercules, CA). Human squamous carcinoma A431 cells were used as positive controls. For negative control, samples were treated with DNase-free pancreatic RNase at a concentration of 1 μg/μl for 20 min at 80°C prior to the TRAP assay.

Results

Chemical transformation of HaCaT cells by MNNG and TCDD

In order to investigate neoplastic transformation of HaCaT cells, the method of chemical transformation was used with MNNG and TCDD for 3 weeks (Fig. 1). There were no morphological differences between one untreated control and the other experimental groups (MNNG, and TCDD treated group). But each experimental group was showed the difference in the growth rate. The untreated control and MNNG groups grew slowly than TCDD group for culture period. And then the cells were progressively deteriorated and died. However, the HaCaT cells co-treated with MNNG and TCDD were able to grow beyond 20 days after treatment and spontaneously produced the foci from random cell origination. As shown in Fig. 2-E, the control cells (Fig. 2-A) spontaneously produced 2.17 ± 1.52 transformed foci per flask, and the cells treated with MNNG (Fig. 2-B) and TCDD (Fig. 2-C) only

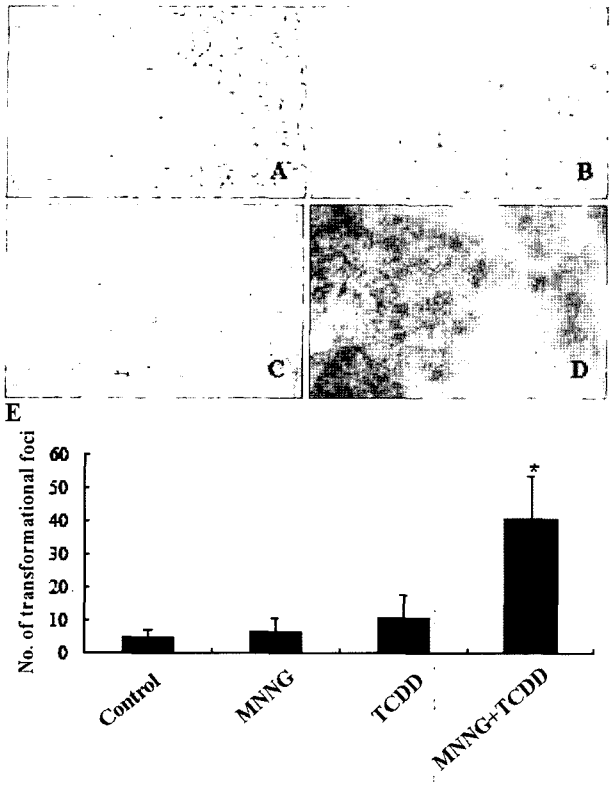

Fig. 2. Effects of TCDD on neoplastic transformation of HaCaT cells. The HaCaT cells were initiated with 0.1 μg/ml MNNG and promoted with 100 nM TCDD for 3 weeks as described in Materials and methods. The cells were visualized with Giemsa staining. Original magnification 40×. A, untreated control group; B, MNNG group; C, TCDD group; D, MNNG-TCDD group; E, Number of transformed foci in four experimental groups. Results of triplicate samples were expressed as mean ± SD. **$p < 0.01$ vs none.

produced about 3.33 ± 2.12 and 6.67 ± 4.83 foci per flask, respectively. In contrast, TCDD treatment after exposure to MNNG markedly enhanced the production of transformed foci at 40.25 ± 13.33 foci per flask which was about 12-fold higher and 6-fold than that of MNNG and TCDD groups, respectively. The MNNG-TCDD co-treatment group was reached a peak confluence of cells per flask on 6 days. The cells of this group were grew more rapidly and began to pile up in random cell origination at 9 days after TCDD treatment in the cells exposure of MNNG for 24 hours. While the cells underwent progressively deteriorated and died, the pile-up cells were went on growth and formed small projections, and released round cells from the focal areas according to the culture periods. Finally, the TCDD-transformed foci were induced at 20 days after TCDD treatment in the cells were initiated with MNNG for 24 hours (Fig. 3). Accordingly, to obtain maximal transformation frequency with MNNG and TCDD, it was necessary to culture the TCDD-treated cells for at least 20 days after exposure of MNNG.

Colony formation of TCDD-transformed HaCaT cells in soft agar

The TCDD-transformed cells were characterized by quantitative differences in growth properties, such as soft agar colony-forming efficiency associated with the neoplastic phenotypes. We tested the cells of four experimental groups including untreated control and TCDD-transformed groups for growth in soft agar.

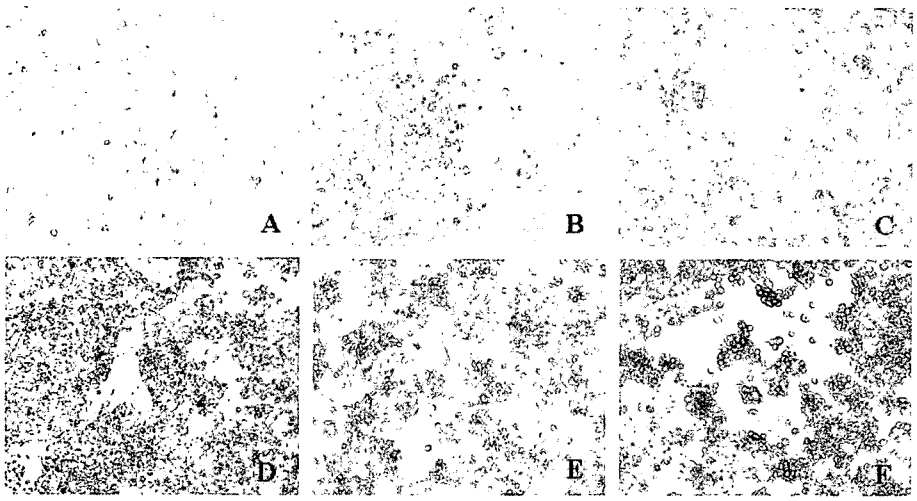

Fig. 3. Morpholgical changes of HaCaT cells by chemical transformation. The HaCaT cells were initiated with 0.1 μg/ml MNNG and promoted with 100 nM TCDD for 3 weeks as described in Materials and methods. Time-dependent changes of HaCaT cells (A, Day 6; B, Day 9; C, Day 12; D, Day 15; E, Day 18; F, Day 21). Original magnification 100×.

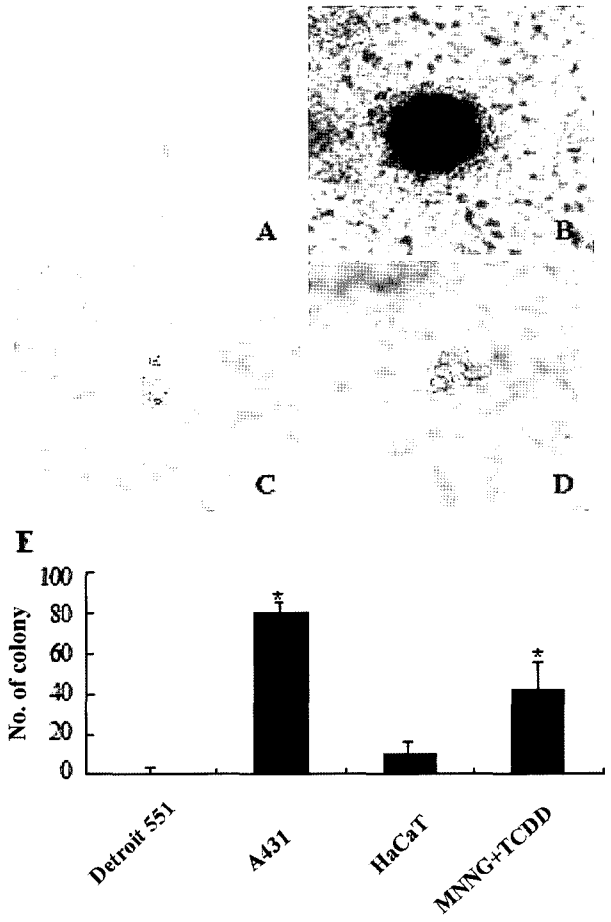

Fig. 4. Effects of TCDD-transformed HaCaT cells on colony formation in soft agar. The TCDD-transformed HaCaT cells were cultured TCDD-transformed cells grew in soft agar for 4 weeks as described in Materials and methods. Original magnification 100×. A, Detroit551 cells; B, A 431 cells; C, untreated control HaCaT cells; D, TCDD-transformed HaCaT cells; E, Number of colony in soft agar. Results of triplicate samples were expressed as mean ± SD. **p < 0.01 vs none.

Untreated control and TCDD-transformed cell suspension are cultured in 0.3% Noble agar for 4 weeks. Colonies were formed in soft agar after 4 weeks (Fig. 4). As shown in Fig. 4-E, in A431 cells, positive control cells, large amount (> 80.50 ± 9.19) of colony formation was observed, whereas, Detroit551 cells, negative control cells, did not grow in soft agar (< 1.00 ± 1.41). Small colonies (fewer than 10 cells) were found in the untreated control cells (10.00 ± 0.71). In the other hands, the TCDD-transformed cells produced about 42.00 ± 8.49 colonies and grew in soft agar with large colonies (more than 20 cells). The large colonies of TCDD-transformed cells grew rapidly to form multilayer

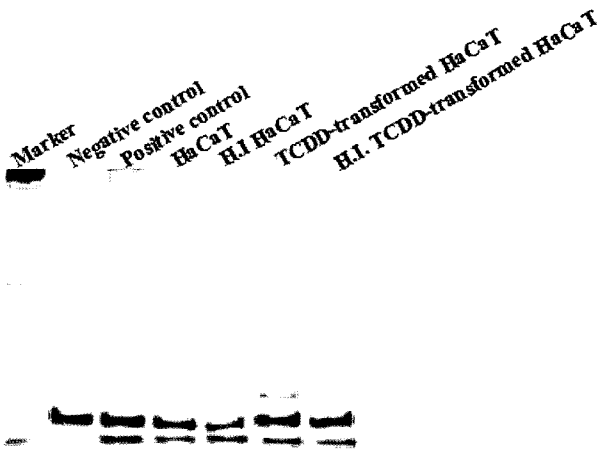

Fig. 5. Relative telomerase activity in TCDD-transformed HaCaT cells. Telomerase activity levels detected with TRAP assay as described in Materials and methods. A431 cells and CHAPS buffer were used as a positive control and negative control for the assay, respectively.

colonies, swelled in the center (Fig. 4-D). It was compacted colonies, and it was difficult to distinguish individual cells and cell-cell boundaries. In contrast, untransformed HaCaT cell line formed less compact colonies than TCDD-transformed cells, so individual rounded cells of each colony were able to distinguish between distinct boundaries (Fig. 4-C)

Telomerase activity of TCDD-transformed HaCaT cells

To reveal whether TCDD-transformed HaCaT cells were changed in telomerase activity, the pattern of telomerase activity in the untreated control and TCDD-transformed cells were examined using TRAP assay (Fig. 5). Untreated control cells were detected in small ladders of telomerase products, but telomerase activity was not detected in heat inactivated HaCaT cells. By contrast, the telomerase activity of TCDD-transformed cells was increased 4- to 5 -fold higher specific activity in parallel measurements than that of untreated control cells.

Discussion

Initiation/promotion studies in skin have demonstrated that TCDD was a potent tumor promoter in mouse skin as well as liver (Poland and Kuntson, 1982). Herbert *et al.* (1990) reported that TCDD promoted the development of papillomas in hairless mouse skin and was more potent than other structural analogues of TCDD

such as Polychlorinated dibenzo-ρ-dioxins (PCDDs) or dibenzofurans (PCDFs). Several studies indicated that TCDD induced neoplastic transformation of mouse fibroblasts and epithelial cells *in vitro* models (Abernethy and Boreiko, 1987; Tanaka *et al.*, 1989). However, TCDD elicited weak biological effects such as chlorance and hyperkeratosis in human epidermal cells (Tayler, 1979). It was indicated that human cells showed a remarkable resistance to transformation by TCDD. Thus, it has been difficult to study the roles of TCDD involved in carcinogenesis of human skin.

In this study, TCDD treatment of human keratinocytes HaCaT cells by initiated with MNNG could promote neoplastic proliferation. Additionally, as determined by TCDD-induced carcinogenesis (Rhim *et al.*, 1985; Yang *et al.*, 1992), TCDD also induced morphological alternations in our cell culture system. These findings suggest that TCDD can act as a potent tumor promoter and causes accelerated transformation in HaCaT cells. Thus, this chemical transformation model using HaCaT cells provides a valuable tool to study on the mechanisms of TCDD-induced carcinogenesis in human skin.

As reported in other studies, TCDD was affected in cellular proliferation by regulation of cytokines or growth factors such as plasminogen activator inhibitor-2 (PAI-2), tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), Interleukin-1β (IL-1β) (Conner *et al.*, 1994; Greenlee *et al.*, 1987; Yang *et al.*, 1999). As noted in this study, neoplastic transformation of MNNG-treated HaCaT cells was induced by TCDD without stimulation by exogenous growth factor or cytokines. It was indicated that TCDD is significantly changed in microenvironment such as cytokines or growth regulatory factors of HaCaT cells. It was not addressed in this study whether the alternation of microenvironment by TCDD eventually led to the neoplastic transformation. Further studies are required to elucidate the alternation of microenvironment by TCDD on the cells.

A number of previous studies have reported that TCDD had induced the inhibition of proliferation and the induction of apoptotic cell death in human cell lines (Hossain *et al.*, 1998; Kikuchi *et al.*, 2001). However, TCDD antagonized the action of apoptosis in primary rat hepatocytes (Woerner and Schrenk; 1998) and altered an aspect of human keratinocyte homeostasis without inducing apoptosis (Loerscher *et al.*, 2001b). Recent studies indicated that TCDD induced the immortalization of normal human ketatinocytes through the suppression of the key initiators of senescence, $p16^{INK4a}$ and p53 (Ray and Swanson, 2004).

In this study, we showed that TCDD alone was sufficient to neoplastic transformation in MNNG-initiated HaCaT cell and telomerase activity increased by TCDD in the transformation cells. Gonzalez-Suarez *et al.* (2000) reported that the absence of telomerase activity has a dramatic inhibitory impact on skin tumorigenesis. It was indicated that tumor formation in skin requires telomere maintenance above a threshold length. Therefore, our findings indicated that the overexpression of telomerase activity results in neoplastic proliferation of MNNG-initiated HaCaT cells in response to TCDD treatment. It may be a way in which telomerase overexpression helps to promote chemical transformation and carcinogenesis in the skin. In addition, this transformation system without stimulation by exogenous growth factors is useful to assess the human toxicity to TCDD. But, telomerase overexoression is able to cooperate with oncogenes such as c-myc, ras, and p53 in cultured immortalized human cells into neoplastic transforming cells (Hahn *et al.*, 1999). Much more works would be needed to elucidate the mechanism of TCDD-induced carcinogenesis in the future.

Collectively, TCDD alone promotes neoplastic transformation in HaCat cells by initiated with MNNG. These transformed HaCaT cells show morphological alternations in soft agar and overexoression of telomerase activity. Therefore, TCDD can act as a potent tumor promoter in human epidermal keratinocytes. Although additional studies are required to understand what extent signal events in cellular transformation system, this chemical transformation model is a good tool in future studies not only to elucidate the mechamism by TCDD-induced carcinogenesis but also to evaluate the effects of xonobiotics on human skin.

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