Dioxin Induces Transforming Growth Factor-α in Human Keratinocytes*

Eui Ju Choi‡§, Diane G. Toscano‡, Judith A. Ryan¶, Norbert Riedell¶, and William A. Toscano, Jr.§**

From the Environmental Toxicology Program, Division of Environmental and Occupational Health, University of Minnesota, Minneapolis, Minnesota 55455, the Diabetes Unit, Massachusetts General Hospital, Boston, Massachusetts 02114, and the Department of Medicine, Boston University School of Medicine, Boston, Massachusetts 02118

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), a widespread environmental toxicant, is a tumor promoter that induces hyperplasia in epithelial cells. Exposure of cultured human keratinocytes to TCDD resulted in a time-dependent dioxin-specific Ah receptor-mediated release of transforming growth factor-α (TGF-α) into the culture medium. Cultures exposed to TCDD showed a rate of TGF-α secretion into the medium of about 30 fmol/ml/day, as well as a 3- to 6-fold increase in TGF-α mRNA expression. Increased production of TGF-α in human keratinocytes exposed to TCDD demonstrates a modulation of autocrine regulation in those cells. These results suggest that induction of TGF-α could be an important part of the mechanism of dioxin-mediated toxicity and tumor promotion.

TCDD is the archetype of the family of related polychlorinated compounds known as dioxins (1). Dioxins are highly toxic by-products formed during the commercial synthesis of certain herbicides and bactericides (2). These compounds exert their toxic actions on cells via a soluble or nuclear receptor known as the Ah receptor that functions in a manner analogous to steroid hormone receptors (1, 3–6). Studies on the potential genotoxicity of TCDD indicate that it is neither a mutagen nor a complete carcinogen because it does not bind directly to DNA (1). Rather, this polychlorinated compound induces hyperplasia (7) and can act as a tumor promoter in the mouse skin tumor promotion assay (8–10). Tumor promoters act by modulating signal transducing pathways that control the proliferation program of cells and thus augment events initiated by a potential carcinogen (11, 12). The mechanisms of hyperplasia and tumor promotion are probably related because both processes involve modulation of the regulators of cell proliferation (12–15).

The EGF receptor plays a pivotal regulatory role in keratinocyte growth (16). In previous studies, we and others (17, 18) reported that exposure of cultured human keratinocytes to TCDD results in the down-regulation of high affinity EGF receptors with a concomitant increase in basal DNA synthesis. TCDD does not bind to the EGF receptor, therefore, its action on the EGF receptor in human keratinocytes could result from an indirect effect on either EGF receptor synthesis or from modulation of autocrine regulation, a process whereby cells produce and secrete their own peptide growth factors (19, 20). Attempts to demonstrate diminished EGF receptor synthesis after dioxin treatment have proven inconclusive (21). Mammalian cells have been shown to produce two distinct growth factors that act as ligands for the EGF receptor, EGF and TGF-α. Keratinocytes synthesize and secrete TGF-α, but not EGF (22). We therefore undertook this study to determine whether human keratinocytes exposed to the dioxin TCDD modified autocrine regulation of TGF-α in these cells.

In this study we used both the clonal keratinocyte cell line, SCC-12F, derived from a squamous cell carcinoma of facial epidermis (23, 24), and primary cultures derived from neonatal foreskin, designated HuE, to assess whether exposure of human keratinocyte cultures to TCDD resulted in altered expression of TGF-α. The SCC-12F cell line is nontumorigenic and responds to the same biochemical regulators of proliferation and differentiation as primary HuE cultures of human keratinocytes and has served as a powerful model system to elucidate a number of toxic mechanisms (17, 25, 26).

MATERIALS AND METHODS

Chemicals—TCDD was obtained from the Stable Isotope Division, ICN, Cambridge, MA. TCDBF and 2,7-DpD were the generous gift of Dr. William F. Greenlee, National Institute of Environmental Health Sciences, Research Triangle Park, NC. Mouse monoclonal anti-TGF-α IgG, anti-EGF receptor IgG, rabbit polyclonal anti-EGF IgG, and protein A-agarose were purchased from Oncogene Science, Inc., Manhasset, NY. TGF-α was obtained from Biotope, Seattle, WA. 32P and 125I were from ICN, Costa Mesa, CA. EGF, purchased from Biomedical Technologies, Stoughton, MA, was iodinated to a specific radioactivity of 75 Ci/mmol using chloramine T (Sigma) and purified by chromatography on Sephadex G-50 (Pharmacia LKB Biotechnology Inc.) according to published methods (27). RQ1-DNase was purchased from Promega, Madison, WI. Multipurpose vector pTT73 was obtained from Pharmacia. All other chemicals were of the highest grade commercially available and were used without further purification.

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§ Travelling student from Harvard University and a fellow of the Albert J. Ryan Foundation.

¶ To whom all correspondence should be addressed: University of Minnesota, Division of Environmental and Occupational Health, Box 197 Mayo Memorial Bldg., 420 Delaware St. S.E., Minneapolis, MN 55455. Tel: 612-626-0184.

⁎ This abbreviations used are: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; DMEM, Dulbecco’s modified Eagle’s medium; Me2S0, methyl sulfoxide; EGF, epidermal growth factor; TCDBF, 2,3,7,8-Tetrachlorodibenzo-furan; 2,7-DpD, 2,7-dichlorodibenzo-p-dioxin; TGF-α, transforming growth factor-α; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; SDS, sodium dodecyl sulfate.

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Cells and Culture Conditions—Primary keratinocyte cultures were routinely prepared using published procedures (28). Briefly, fresh neonatal foreskin was obtained from the circumcision room of local hospitals and immediately placed into DMEM (Hyclone). Subcutaneous tissue was removed, the remaining epidermal tissue was minced and treated with 0.25% trypsin containing 0.1% (150 mM) was added. The mixture was agitated for 30 min at 37 °C and was allowed to settle for 1 min, after which the supernatant containing keratinocytes was placed in 15 ml of DMEM containing bovine serum (10%). After centrifugation for 5 min at 800 g in a clinical centrifuge, the supernatant containing the trypan was removed and the cells were suspended in DMEM containing 5% fetal bovine serum (Hyclone), 12 ng/ml EGF, 9 ng/ml cholera toxin, and 400 ng/ml hydrocortisone. The suspended cells were placed on a 100 mm tissue culture dish containing lethally irradiated murine 3T3 fibroblasts. SCC-12F cells were the generous gift of Dr. James Rheinwald of the Dana Farber Cancer Institute, Boston. Cells were grown in DMEM supplemented with 5% fetal calf serum (Hyclone) on a feeder layer of lethally irradiated murine 3T3 fibroblasts (29, 30). The cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO2. Cells cultivated in this manner typically reach confluence after 10 days. The 3T3 fibroblasts were removed from keratinocyte cultures by rinsing the culture dishes with 0.02% EDTA in phosphate-buffered saline. A431 cells, a gift from Dr. Laurie Hudson, Northwestern University Medical School, Chicago, IL (ATCC CRL 1555) were cultivated in DMEM supplemented with 5% bovine serum albumin (31).

Exposure of Keratinocyte Cultures to Bis(oxazolines)—When cells reached confluence, the cultures were rinsed with serum-free DMEM and exposed to either TCDD or 0.1% Me2S0 (15 mM) in the presence of 100 nM EGF. After exposure for the various times indicated in the figures, the concentrations of TGF-α in the culture medium were calculated using a C-fit program (33). Immunoprecipitation of EGF Receptor—Membranes from A431 cells were solubilized for 1 h at 4 °C in solubilizing buffer (10 mM sodium phosphate buffer, pH 7.2, containing 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 30 mM NaF, and 0.1% bovine serum albumin) and subjected to centrifugation at 30,000 × g for 1 h. One ml of the solubilized fraction (1 mg/ml) was exposed to either TCDD or 0.1% Me2S0 in serum-free DMEM. After exposure for the various times indicated in the figures, the culture medium was collected. When necessary, the collected medium was dialyzed against H2O to lower the salt concentration, and concentrated by lyophilization.

Receptor Binding Assay—In whole cell receptor binding experiments, fresh confluent cultures of SCC-12F cells in six-well 35-mm dishes were incubated for 4 h at 4 °C in binding buffer (DMEM, 20 mM HEPES, pH 7.4, and 0.1% bovine serum albumin (Hyclone) containing 10 nM EGF (50 pm). The binding reaction was terminated by rinsing the cultures five times with ice-cold balanced salt solution and then the cultures were solubilized by adding 0.1 N NaOH. 125I in the solubilized extract was quantified using a Packard Cobra γ-counter. Nonspecific binding was determined in the presence of 100 nM EGF. In sequential competitive receptor binding assays, fresh cultures of SCC-12F in 35-mm 6-well dishes were incubated for 6 h at 37 °C with medium (1 ml) from either TCDD-treated or control cultures. After the incubation, the cultures were washed four times with ice-cold binding buffer and incubated with 125I-EGF (50 pm) in binding buffer for 4 h at 4 °C. 125I-EGF binding to the cultures was measured as described above. For competitive receptor binding assays, membranes from A431 cells were prepared using published procedures (32). Aliquots of A431 membranes (3 μg of protein) and 125I-EGF (10-50 pm) were incubated at 37 °C for 60 min in 20 mM HEPES, pH 7.4, containing bovine serum albumin (1 mg/ml), and unlabeled EGF standards (50-500 pm) or conditioned medium (200 μl, ~0.1 mg protein) in a final volume of 250 μl. Nonspecific binding was determined in the presence of excess unlabeled EGF (200-fold) and was typically 5-10% of total EGF binding. The incubation was terminated by dilution with 2 ml of ice-cold 20 mM HEPES, pH 7.4, containing bovine serum albumin (1 mg/ml). Bound 125I-EGF was separated from free radioligand by centrifugation at 30,000 × g for 30 min. The supernatant was removed and radioactivity in the pellet was quantified using a Packard Cobra γ-counter. B/B values and the concentration of TGF-α in the conditioned medium were calculated using a C-fit program (33).

Immunoblotting for TGF-α—Samples of lyophilized medium were suspended in 20 mM phosphate, pH 7.2, and were subjected to electrophoresis on 15% polyacrylamide gel in the presence of 1% NaDoDSO4. After electrophoresis, proteins were transferred onto nitrocellulose membranes using an electrobbonitting apparatus (Bio-Rad) and blocked with 5% bovine serum albumin (20 g/ml Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). The nitrocellulose membranes were incubated with monoclonal mouse anti-TGF-α (5 μg/ml in TBST), washed twice with the same buffer, and incubated with alkaline phosphatase conjugated goat anti-mouse IgG (30 μg/ml in TBST) for 1 h. The blot was washed again and reacted with alkaline phosphatase substrate solution (100 mM NaF, 50 mM NaCl, 1.2 mM MgCl2, 0.2 mg/ml nitroblue tetrazolium, and 0.1 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate). The reaction was terminated by washing the nitrocellulose membranes with H2O.

Immunoprecipitation of EGF Receptor—Membranes from A431 cells were solubilized for 1 h at 4 °C in solubilizing buffer (10 mM sodium phosphate buffer, pH 7.2, containing 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 30 mM NaF, and 0.1% bovine serum albumin) and subjected to centrifugation at 30,000 × g for 1 h. One ml of the solubilized fraction (1 mg/ml) was exposed to either TCDD or 0.1% Me2S0 in serum-free DMEM. After washing with 1 ml of mouse monoclonal anti-EGF receptor IgG (Oncogene Science, Inc.) and protein A-agarose at 4 °C overnight. Immunoprecipitate was recovered by centrifugation at 4,000 × g for 10 min and washed four times with solubilizing buffer. The EGF receptor immunocomplex was phosphorylated and examined by polyacrylamide gel electrophoresis as described below.

Phosphorylation of EGF Receptor from A431 Cell Membranes—The EGF receptor immunoprecipitate or protein from solubilized A431 cell membranes was phosphorylated as described above, except unlabeled ATP (140 μM) was substituted for [γ-32P]ATP. After adding 2 × Laemmli's sample buffer, the mixture was heated for 2 min at 100 °C and subjected to electrophoresis on 5% polyacrylamide gel in the presence of SDS. 32P-Labeled polypeptides bands on the gel were visualized by autoradiography. The positive control consisted of the EGF receptor immunoprecipitate or the solubilized A431 cell membranes incubated with 10 nM EGF.

Immunoblotting for Phosphotyrosine—Solubilized A431 cell membranes were phosphorylated as described above, except unlabeled ATP (140 μM) was substituted for [γ-32P]ATP. After adding 2 × Laemmli's sample buffer, the mixture was heated for 2 min at 100 °C and subjected to electrophoresis on 5% polyacrylamide gel in the presence of SDS. The polypeptides separated on the gel were transferred to nitrocellulose membranes using an electrobbonitting apparatus. After blocking with bovine serum albumin, the nitrocellulose membranes were incubated with a polyclonal antiphosphotyrosine IgG (35), washed with TBST buffer, and incubated with 125I-protein A. The 125I-labeled blots specific for phosphotyrosine were visualized by autoradiography, and quantified by densitometry.

Protein Assay—Protein was estimated using the procedure of Bradford (36)).

FIG. 1. Decrease of 125I-EGF binding as a function of secreted growth factor. Fresh confluent cultures of SCC-12F cells in 35-mm dishes were incubated for 4 h at 4 °C in 1 ml of binding buffer, containing 125I-EGF and the indicated volume of TCDD (100 nM)-conditioned medium (200 μl, ~0.1 mg protein) in a final volume of 250 μl. Nonspecific binding was determined in the presence of excess unlabeled EGF (200-fold) and was typically 5-10% of total EGF binding. The incubation was terminated by dilution with 2 ml of ice-cold 20 mM HEPES, pH 7.4, containing bovine serum albumin (1 mg/ml). Bound 125I-EGF was separated from free radioligand by centrifugation at 30,000 × g for 20 min. The supernatant was removed and radioactivity in the pellet was quantified using a Packard Cobra γ-counter. B/B values and the concentration of TGF-α in the conditioned medium were calculated using a C-fit program (33).
Dioxin Modulation of TGF-α

Effect of dioxin concentration on EGF binding and the concentration of EGF-receptor competing factor in the culture medium. Medium from confluent HuE or SCC-12F cultures was exposed to TCDD, TCDBF at the concentrations shown above, or 2,7-DpD (1 μM) in serum-free medium for 4 days. Control cultures were exposed to 0.1% MeSO under the same conditions. Conditioned medium was collected as described under “Materials and Methods.” A, modulation of EGF receptor binding as a function of dioxin concentration. Sequential competitive EGF receptor binding assay was performed using fresh confluent cultures of SCC-12F cells in 6-well 35-mm dishes as described under “Materials and Methods.” Cholera toxin (9 ng/ml) and hydrocortisone (400 ng/ml) were present in the binding assays using medium from control or dioxin exposed HuE cultures. The results shown above represent the mean of triplicate determinations and are expressed as percent [125I]-EGF bound, relative to their respective MeSO controls. Control values for HuE and SCC-12F (100% binding) were 52.2 ± 0.8 and 32.1 ± 0.2 fmol of [125I]-EGF bound per mg of protein, respectively. The binding observed in assays containing medium from HuE and SCC-12F cultures exposed to 2,7-DpD (1 μM) was 50.7 ± 6 (97%) and 31.6 ± 0.4 (98%) fmol of [125I]-EGF bound per mg of protein, respectively. Medium from SCC-12F cultures exposed to TCDD (□); medium from SCC-12F cultures exposed to TCDBF (●); medium from HuE cultures exposed to TCDBF (●). Lowry as modified by Peterson (36). Bovine serum albumin was used as the standard.

Preparation of Total Cellular RNA from HuE and SCC-12F Cells—For the preparation of total cellular RNA, cells were washed and immediately lysed in guanidinium isothiocyanate, followed by extraction of RNA using standard protocols (37). The resuspended RNA was submitted to RQ1-DNase (Promega Biotec) digestion to remove residual DNA, extracted twice with an equal volume of phenol/chloroform, precipitated, dissolved in diethylpyrocarbonate-treated H2O containing 0.1% SDS, and quantified by measuring the absorbance at 260 nm.

Subcloning and Purification of the TGF-α Insert—A 917-bp pair human TGF-α cDNA (38) cloned into plasmid vector pBR322 (pHTGF 1-10925) was kindly provided by Dr. S. Farmer (Boston University Medical School) with permission of Dr. Graeme Bell (Howard Hughes Medical Institute, University of Chicago). The EcoRI insert was subcloned into the multipurpose vector pT7T3 (Pharmacia), amplified, excised, and purified by electrophoresis. The purified insert was quantified, and aliquots of 100 ng were random prime labeled (Promega) in the presence of [α-32P]dCTP to specific activities of approximately 2 × 10^6 cpm/μg insert.

Northern Blot Analysis—Aliquots of RNA (10 μg) were electrophoretically separated on denaturing 1% agarose, formaldehyde gels and stained with ethidium bromide to examine the quality and quantity of RNA. Gels were equilibrated in 20 × SSC (3 M sodium chloride, 300 mM sodium citrate) and the RNAs were transferred onto nylon filters (ICN). Filters were baked at 80 °C for 2 h, prehybridized for 16 h, and hybridized with 10^6 cpm/μl of the human TGF-α cDNA insert for 48 h in a mixture containing 50% formamide, 10% dextran sulfate, 800 mM sodium chloride, 100 mM MOPS/0.1% sodium laurel sarcosine/denatured herring sperm DNA (100 μg/ml)/and 0.6% each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll (39).

After hybridization, filters were washed once at room temperature in 5 × SSC (750 mM NaCl, 0.07 mM sodium citrate) plus 0.05% sodium pyrophosphate and 0.1% sodium laurel sarcosine, followed by two washes at 56 °C in 0.2 × SSC (30 mM NaCl, 3 mM sodium citrate) plus 0.05% sodium pyrophosphate and 0.1% sodium laurel sarcosine and one wash at 56 °C in 0.1 × SSC (15 mM NaCl, 1.5 mM sodium citrate) plus 0.05% sodium pyrophosphate and 0.1% sodium laurel sarcosine. Filters were dried and exposed to Kodak XAR film at −70 °C for 21 days. Filters were subsequently hybridized with an actin probe to calibrate the amount of hybridizable RNA, and the intensity of hybridization bands was measured by scanning densitometry.

RESULTS

To test whether exposure of human keratinocytes to TCDD modulated autocrine regulation by stimulating the production of TGF-α, we examined medium from both primary (HuE) and immortalized non-tumorigenic human keratinocyte cultures (SCC-12F) for the presence of a factor that would modify EGF binding to its receptor. [125I]-EGF binding to EGF receptors in fresh SCC-12F keratinocyte cultures was attenuated by medium from both HuE and SCC-12F cultures that had been exposed to TCDD (HuE = 64.0 ± 0.3 versus 21.1 ± 0.8 and SCC-12F = 28.2 ± 1.0 versus 17.9 ± 0.3 fmol of [125I]-EGF bound/mg protein ± S.E. in control medium versus medium from six different HuE and SCC-12F cultures exposed to 10 and 100 nM TCDD, respectively). The apparent loss of approximately 40% of EGF-binding in the keratinocyte cultures

![Graph A](image1.png)

![Graph B](image2.png)
was dependent on the concentration of dioxin-conditioned keratinocytes exposed to TCDD (17, 40). Competition of the dioxin-induced factor with $^{125}$I-EGF for binding to the EGF receptor was dependent on the concentration of dioxin to which the cells were exposed (Fig. 2A). The EC$_{50}$ of about 2 nM for TCDD was in excellent agreement with the EC$_{50}$ (half-maximal effective concentration) value obtained in our earlier studies for down-regulation of EGF receptors in keratinocyte cultures exposed to TCDD (17). To assess further whether the dioxin-specific Ah receptor mediated the lowered EGF binding, we examined EGF-binding in cultures exposed to medium from human keratinocytes exposed to various concentrations of TCDBF and a high concentration (1 µM) of 2,7-DpD. In this study, keratinocyte cultures exposed to TCDBF, an active congener of dioxin that exerts an Ah receptor-mediated toxic response (41), yielded medium that reduced $^{125}$I-EGF binding in fresh cultures. In contrast, 2,7-DpD, an inactive analog of TCDD, did not affect EGF binding even at concentrations 10 times that of TCDD. Our data suggested the possibility that a ligand specific for the EGF receptor, such as TGF-α, was present in the medium of human keratinocytes exposed to active dioxin congeners. On the assumption that the putative factor competed with EGF for the EGF receptor with an approximately equivalent dissociation constant, we examined the concentration of the factor using EGF-receptor competitive binding assays in A431 membranes. The data shown in Fig. 2B indicate a dioxin concentration-dependent increase in the putative competing factor in medium from dioxin-exposed keratinocyte cultures. These data taken together suggest a possible role for the TCDD receptor as a modulator of TGF-α expression in human keratinocytes exposed to dioxins.

Immunoblot analysis using a monoclonal anti-TGF-α, that does not recognize EGF, demonstrated that exposure of human keratinocyte cultures to TCDD markedly enhanced the expression of TGF-α in the medium of those cells (Fig. 3). A strong band of TGF-α (molecular mass = 6 kDa) was detected in medium from HuE cultures exposed to TCDD but not in medium from control cultures. Similar results were obtained with SCC-12F cells (Fig. 3). Western blot analysis for EGF indicated that serum free medium from both control and dioxin-exposed cultures did not contain immunoreactive EGF. The concentration of TGF-α found in serum-free medium increased with time of exposure of the cultures to TCDD (Fig. 4). Increased levels of TGF-α in the medium from both HuE and SCC-12F cells could be detected after cultures were exposed to the dioxin for at least 24 h. The rate of appearance of TGF-α in the medium was about 30 fmol/ml of medium/day. The tonic level of TGF-α in the culture medium from control cells remained relatively constant during the incubation.

Increased levels of TGF-α in the medium of cultures exposed to TCDD could result from either increased synthesis or the secretion of previously synthesized peptide. To determine whether TGF-α mRNA levels were altered as a consequence of dioxin exposure, we performed Northern blot analysis for TGF-α mRNA. HuE and SCC-12F cultures were exposed to TCDD (10 and 100 nM, respectively) for 96 h. Control experiments were carried out on HuE and SCC-12F cells that had been exposed to Me$_2$SO (0.01 and 0.1%, respectively). TGF-α mRNA levels increased markedly in both HuE and SCC-12F cells as a function of time of exposure to TCDD (Fig. 3). The concentration of TGF-α was estimated in serum-free medium from SCC-12F and HuE cells using the radioreceptor assay described under "Materials and Methods." A, HuE cultures: control medium (○); medium from TCDD-treated (10 nM) cells (●). B, SCC-12F cultures: control medium (○); medium from TCDD-treated (100 nM) cells (●).
SCC-12F cultures (3- and 6-fold, respectively) after exposure to TCDD (Fig. 5). These data strongly suggest that TCDD modulated TGF-α gene expression either transcriptionally or posttranscriptionally in human keratinocytes.

The above results indicated that TCDD altered the expression of both TGF-α and TGF-β mRNA. To test whether TGF-α secreted from TCDD-exposed keratinocyte cultures was biologically active, we examined the action of conditioned medium on phosphorylation of the EGF receptor, a well characterized response of the EGF receptor to TGF-α (42). Membranes from A431 human epidermoid carcinoma cells were used in these experiments because they contain an inordinately high number of EGF receptors. Medium from cultures exposed to TCDD enhanced phosphorylation of the EGF receptor (molecular mass = 170 kDa) about three times that of control medium (Fig. 6A). Experiments using anti-phosphotyrosine IgG confirmed that enhanced phosphorylation of the EGF receptor occurred at tyrosine residues (Fig. 6B). Specificity of the medium-modulated phosphorylation of the EGF receptor was further confirmed by examining phosphorylation of the receptor after immunoprecipitation of the EGF receptor from A431 membranes using rabbit polyclonal anti-EGF receptor IgG (Fig. 6C). Taken together, these observations corroborate evidence that TCDD treatment of both SCC-12F and HuE cells increased secretion of a functionally active TGF-α.

**DISCUSSION**

High affinity EGF receptors comprise approximately 1% of the total EGF receptor population in the human keratinocytes used in the present study (2.3 × 10⁵ high affinity sites and 2.0 × 10⁵ low affinity EGF sites/cell (17)). Previous studies from this laboratory (17) showed that exposure of human keratinocytes to TCDD results in down-regulation of high affinity EGF receptors with a concomitant increase in basal DNA synthesis. The lowered number of EGF receptors after TCDD exposure does not result from a decrease in EGF receptor biosynthesis (21). The present study demonstrated that TGF-

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**Fig. 6.** Phosphorylation of the EGF receptor of A431 cells in the presence of medium from control and TCDD-exposed SCC-12F cultures. A, solubilized membranes of A431 cells (44 μg) were phosphorylated in the presence of concentrated medium (7.5 μg of protein) from TCDD (100 nM)-exposed (lane 2) or control cultures (lane 3) and subjected to electrophoresis on 5% polyacrylamide gel, as described under "Materials and Methods." The positive control consisted of A431 cell membranes incubated with 10 nM EGF (lane 1). Each lane of the gel contained 10 μg of solubilized protein from A-431 cells. B, tyrosine-specific phosphorylation of the EGF receptor was analyzed by an immunoblot using a polyclonal anti-phosphotyrosine IgG and 125I-protein A as described under "Materials and Methods." Each lane of the gel contained 15 μg of protein. 125I-labeled proteins were visualized by autoradiography and quantified by densitometry: lane 4 (control) = 310 area units and lane 5 (TCDD-treated) = 876 area units. C, EGF receptor from A431 cell membranes was immunoprecipitated and phosphorylated in the presence of concentrated medium from TCDD-exposed cultures (lane 1), control cultures (lane 2), buffer (lane 3), or 10 nM EGF (lane 4). Each lane of the gel contained the entire immunoprecipitate, approximately 500 ng of protein.

α was overexpressed in cultured human keratinocytes exposed to TCDD. The apparent loss of EGF receptors in the presence of medium from dioxin-exposed cultures evidently occurs because of occupation of the EGF receptor by TGF-α produced by those cells and correlates well with our previous studies indicating the loss of all high affinity EGF binding sites and stimulation of DNA synthesis under these conditions (17). Occupation of the high affinity EGF receptors by a ligand such as TGF-α is an important step in EGF-receptor action (43-45). Both EGF and TGF-α specifically prolong the lifespan of keratinocytes (46) by stimulating lateral migration of proliferating keratinocytes in expanding colonies (47). The appearance of TGF-α in the medium of cells exposed to various concentrations of the active dioxin congeners, TCDD or TDBF, and the absence of TGF-α in medium from keratin-
ocytes exposed to the inactive chlorinated compound, 2,7-DpD at high concentration, suggested that this action of dioxin was mediated in some manner by the TCDD receptor. 

Primary cultures of normal human keratinocytes are capable of producing TGF-α in growth-stimulating conditions and the addition of TGF-α to those cultures results in the autostimulation of TGF-α biosynthesis (48,49). Furthermore, TGF-α stimulates DNA synthesis and the clonal growth of cultured keratinocytes (50,51). Epidermal keratinocytes do not synthesize EGF (22). These observations imply that the natural physiological factor that activates the EGF receptor in human keratinocytes is TGF-α. The present study showed that the level of TGF-α mRNA expressed in unexposed cultures of HuE and SCC-12F cells was negligible. Our results are in agreement with published studies (48,52) showing that cultures of normal human keratinocytes in medium lacking growth stimulating factors do not secrete TGF-α. The data suggest that the induction of TGF-α expression is tightly controlled in human keratinocytes under normal conditions. Exposure of keratinocytes to TCDD results in the unregulated or misregulated secretion of TGF-α in those cells. One possible mechanism by which TCDD alters the cellular control of TGF-α production could be that the TCDD-bound Ah receptor enhances the transcription of TGF-α gene. It has been proposed that the ligand-bound Ah receptor acts as a transcription stimulating factor for cytochrome P450 gene and other genes (5,53).

Previous studies of epidermal cells both in vivo and in vitro have suggested that many of the biological responses resulting from exposure to TCDD are similar to those found after TGF-α or EGF treatment. These include tumor promotion (9,10,54,55), early eyelid opening (56-58), hyperplasia of epidermal keratinocytes and other epithelial tissues (1,59,60), and epidermal keratinization (61,62). The similarities between the action of TCDD and those of EGF or TGF-α on epidermal cells strongly support the conclusion that overexpression of TGF-α could be an important mechanism for the toxicity resulting from exposure to TCDD and other dioxins. It is not clear whether other cell types can also produce TGF-α in response to TCDD exposure. There are, however, some observations implying that hepatocytes and thymocytes might also produce TGF-α or EGF after exposure to TCDD (57,63,64). Liver and thymus are among tissues susceptible to dioxin toxicity. Studies from other laboratories have shown that EGF binding to the EGF receptor of murine hepatocytes was diminished after TCDD was administered in vivo (57). Tyrosine-specific protein phosphorylation was also stimulated in murine thymus and liver cells after TCDD treatment (63,64). Although these studies did not examine specific protein kinases, those observations are consistent with the model of TGF-α induction by TCDD.

Autocrine regulation is a growth controlling mechanism in which cells secrete their own growth factors that in turn modulate the growth of the secreting cells. Autocrine regulation of cell growth was initially proposed as a mechanism for altered growth regulation of tumorigenic cells (19). Human keratinocytes have an autocrine mechanism for regulating their own growth. Primary cultures of human keratinocytes have both high affinity and low affinity EGF receptors (65) and produce TGF-α when their growth is stimulated by serum or other factors (48), suggesting that TGF-α regulates the proliferation of normal human keratinocytes through an autocrine system and therefore produce the growth factor only when it is required. In the present study, exposure of cultured human keratinocytes to TCDD resulted in the continuous production of TGF-α. The excess TGF-α production could perturb the tightly regulated autocrine system of TGF-α, resulting in an altered growth pattern for epithelial keratinocytes. Overproduction of TGF-α could play a crucial role in the tumor-promoting actions of TCDD. Cells initiated by a carcinogen would continuously secrete TGF-α after exposure to TCDD, resulting in growth stimulation of the initiated cells that could progress to neoplastic tumors. Interestingly, a decrease of EGF binding to EGF receptors appears to be a common response of cells to several different types of tumor promoters. Those tumor promoters include not only phorbol esters, but also compounds such as palytoxin, saccharin, and cyclamate that act independently of protein kinase C (66-68). It is tempting to propose that the induction of TGF-α may be the common mechanism for the down-modulation of the EGF receptor and tumor promotion by diverse tumor promoters. In fact, it was reported recently that cultured epidermal keratinocytes exposed to TPA also secreted TGF-α (51).

Based on the observations in the present study, we are proposing that TCDD, acting through the Ah receptor, deranges signal transduction systems that regulate cell proliferation and differentiation by inducing the overexpression of TGF-α. The overproduced TGF-α alters the control of normal cellular growth patterns by both an autocrine mechanism within the target cell and paracrine effects on neighboring cells. This proposed mechanism suggests that the induction of TGF-α may play a pivotal role in TCDD-mediated hyperplasia, tumor promotion, and other toxic manifestations.

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