2,3,7,8-Tetrachlorodibenzo-p-dioxin-dependent Regulation of Transforming Growth Factors-α and -β2 Expression in a Human Keratinocyte Cell Line Involves Both Transcriptional and Post-transcriptional Control*

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2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), a potent modulator of epithelial cell growth and differentiation, has been shown to induce transforming growth factor (TGF)-α in cultures of human keratinocytes and in the human keratinocyte cell line, SCC-12F. In this report, we investigated the mechanisms by which TCDD alters TGF-α expression. In addition, we studied the actions of TCDD on TGF-β1 and TGF-β2 expression. Treatment of SCC-12F cells with TCDD resulted in an increase in TGF-α and a reduction in TGF-β2 mRNA levels while mRNA levels for TGF-β1 were unchanged. Changes in TGF-α and TGF-β2 expression were maximal by 24 h. No change in the rate of transcription of TGF-α was detected following treatment with TCDD as determined by nuclear run-off analysis. TCDD treatment resulted in a stabilization of TGF-α mRNA as judged by an approximately 2-fold higher level of TGF-α mRNA in treated versus control cells in the presence of actinomycin D. In contrast to TGF-α, the rate of transcription of TGF-β2 was significantly reduced following TCDD treatment. These findings demonstrate that the induction of TGF-α expression in SCC-12F cells by TCDD occurs post-transcriptionally, primarily by mRNA stabilization, while TGF-β2 expression is reduced due to a decrease in the rate of TGF-β2 gene transcription.

TCDD,1 a potent and widespread environmental contaminant, has been associated with tumor promotion and carcinogenesis in rodents (for reviews, see Refs. 1 and 2). In humans, the most commonly observed adverse response to TCDD is chloracne, a skin disorder characterized by a pattern of pathological changes, the most striking of which is squamous metaplasia of epithelial cells within the duct of the sebaceous gland. This is accompanied in some individuals by interfollicular epidermal hyperkeratinization (3). Altered programming of cell growth and differentiation is essential for the pathogenesis of chloracne and can be modeled in vitro using cultures of human epidermal keratinocytes and cell lines derived from squamous cell carcinomas (SCC) (4-7). Treatment of cultured human epidermal cells with TCDD results in an increase in the number of cells undergoing terminal differentiation (4, 6, 8). The SCC-12F cell line used in these studies, a nontumorigenic keratinocyte cell line derived from an SCC of the face (9), also responds to TCDD with an increase in the number of cells undergoing terminal differentiation (6).

Many of the actions of TCDD have been shown to be mediated through an intracellular binding protein, designated the Ah receptor (10). In most cells the Ah receptor mediates the TCDD-dependent expression of a battery of genes that includes cytochrome P450 (CYP1A1) (10, 11). Transcriptional activation of CYP1A1 is a primary response to TCDD resulting from the binding of the TCDD-Ah receptor complex to specific enhancer regions upstream of the CYP1A1 transcription start site (11, 12). In certain cells responsive to TCDD, including human keratinocytes, the TCDD-Ah receptor complex is postulated to alter the expression of genes involved in growth control (6, 13).

Transforming growth factors, originally identified by their ability to stimulate growth of rodent fibroblasts in soft agar (14, 15), have since been found to affect the growth and differentiation of a number of different cell types (16-18), including keratinocytes (19, 20). Transforming growth factor-α (TGF-α) shares structural homology with epidermal growth factor (EGF), utilizes the same receptor as EGF, and possesses a nearly identical spectrum of activity (16, 21). The TGF-βs are a family of related proteins which are structurally and biologically distinct from TGF-α (17, 22, 23). Both TGF-α and the TGF-βs are thought to act as autocrine regulators of keratinocyte proliferation and differentiation (20, 24-26). TGF-α is mitogenic for keratinocytes, while TGF-β1 and TGF-β3 are reported to inhibit keratinocyte proliferation (17-19).

Recently Choi et al. (27) reported that treatment of cultured human keratinocytes or SCC-12F cells with TCDD resulted in an increase in the levels of TGF-α mRNA and secreted TGF-α peptide. They suggested that overexpression of TGF-α is an important mechanism by which TCDD produces its effects. We report that the increase in TGF-α mRNA in SCC-12F cells following TCDD treatment results from a stabilization of the TGF-α mRNA. In addition, we show that TCDD decreases the level of TGF-β2 mRNA by a mechanism involving transcriptional control of TGF-β2 gene expression.
EXPERIMENTAL PROCEDURES

Cells and Culture Conditions—SCC-12F cells (provided by Dr. J. Rheinwald, Biosurface Technology, Inc., Cambridge, MA) were plated in plastic culture dishes (Falcon Plastics, Oxnard, CA) at 2.5 x 10^4 cells/cm^2 in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA) supplemented with 5% fetal calf serum (Hyclone, Logan, UT), plus a final concentration of 40 mM L-glutamine and 1 mM sodium pyruvate and incubated at 37 °C in a humidified atmosphere of 5% CO2:95% air. We have found that addition of fresh media as serum altered the expression of many of the genes which we were investigating. To avoid this effect we do not add fresh media at the time of TCDD treatment. After incubation for 48 h, media was removed and pooled. To the pooled media, TCDD (KOR Isotopes Inc., Cambridge, MA) in MeSO (Sigma) or 0.1% MeSO as control was added, and the media was then aliquotted back into the cultures.

Determination of Cell Number and Colony Formation Efficiency—Cell number was determined in triplicate for control and TCDD-treated cultures in 12-well plates using a Coulter counter (Coulter Electronics, Inc., Hialeah, FL). Colony formation efficiency was determined by plating cells in 5 ml of Dulbecco's modified Eagle's medium + 5% fetal calf serum in triplicate in 60-mm dishes (5000 cells/dish).

For incubation with TCDD, cultures in 12-well plates were treated with 10 nM TCDD. After incubation for 1 week, cells were washed once in phosphate-buffered saline, fixed in methanol, and stained with 10% Giemsa (J. T. Baker, Inc., Phillipsburg, NJ). Colony numbers were determined and results expressed as percent of colonies formed per total number of cells plated. Colonies were counted if they contained 10 or more cells.

Cross-linked Envelope Assay—Cells were collected by trypsinization, resuspended in 5 ml of Leibovitz L-15 medium (GIBCO-BRL); split into 2 tubes with 10^6 cells/tube, spun down, and resuspended in 0.5 ml L-15 medium. Spontaneous envelopes were determined by adding 0.5 ml of lysis buffer (50 mM dithiothreitol, 0.5% SDS) to the first tube and counting the number of envelopes/ml with a hemacytometer. Competent envelope formation was determined by adding 2.5 μl of Ca^2+ ionophore A23187 (Sigma, 1 mg/ml in MeSO) and incubating with mixing for 3-5 h at 37 °C. After incubation, the procedure followed was the same as with spontaneous envelope formation.

Northern Analysis—Cells were collected by scraping and mRNA was isolated using the procedure supplied with the FastTrack mRNA isolation kit (Invitrogen, San Diego, CA). mRNA was precipitated in 2.5 volumes of ethanol, resuspended in water, and quantified. For Northern analysis, mRNA (5 μg/sample) was denatured with glyoxal, size separated by agarose gel electrophoresis, and transferred to a nylon membrane (Bio-Rad). Hybridization of RNA with the indicated DNA probes was carried out overnight at 42 °C in a solution of 50% formamide, 5 × SSSPE (0.15 M sodium phosphate, 1 mM EDTA), 5 × Denhardt's, 0.5% SDS, and 100 μg/ml salmon sperm DNA. The presence of specific RNA was detected by autoradiography and quantified by densitometry. After quantification, filters were stripped and reprobed with each of the indicated DNA probes. A glyceraldehyde phosphate dehydrogenase DNA probe was used as a loading and transfer control to ensure equal quantities of RNA in control and TCDD-treated samples. To confirm results obtained with glyceraldehyde phosphate dehydrogenase, an actin DNA probe was used as a secondary loading and transfer control. Results obtained with actin were similar to those obtained with glyceraldehyde phosphate dehydrogenase. Experiments were run 2-5 times with different RNA preparations.

Plasmids containing DNA for TGF-α (pHTGF1-10-3350) and TGF-β (pHTGFBE-2) were obtained from ATCC (Rockville, MD). Involutin (28), TGF-β2 (29), and glyceraldehyde phosphate dehydrogenase (30) DNAs were obtained from Dr. Cheryl Walker (Chemical Induction Institute of Toxicology, Research Triangle Park, NC). The DNA probe for CYP1A1 (cytochrome P-450) (31) was a gift from Dr. Robert Tukey (University of California, La Jolla, CA).

TGF-α mRNA stabilization Assay—Cells were plated and treated as described above. After incubation for 12 h, media was removed from control or TCDD-treated cultures and pooled. Actinomycin D (15 μg/ml, Sigma) was added to the pooled media and the media was replaced with the cultures. This concentration of actinomycin D inhibited [3H]uridine incorporation by 90-95% (data not shown). At the indicated times after addition of actinomycin D, RNA was collected for Northern analysis as described above. Briefly, nuclei collected by Nonidet P-40 lysis were added directly to 2 x reaction buffer (20 mM Tris (pH 8), 20% glycerol, 140 mM KCl, 10 mM MgCl₂, 1 mM MnCl₂, 14 mM 2-mercaptoethanol, 1 mM ATP, CTP, and GTP, 60 mM phosphocreatine, 200 units of creatine kinase, 0.8 mM phenylmethylsulfonyl fluoride, 8 mM dithiothreitol, 0.8 units/μl RNase (Promega, Madison, WI), and 250 cCi of [3P]UTP (800 Ci/mmol, Amer sham Corp.) and incubated for 20 min at 25 °C. The nascent transcripts were processed for hybridization by the hot phenol method as described by Nevin (32). An equal amount of radiolabeled RNA was added to DNA immobilized on nylon filters and hybridized for 3 days at 42 °C in 50% formamide hybridization buffer prepared as described above. Filters were prepared by adding 5 μg of alkali-denatured plasmid per slot and UV cross-linked with a UV Stratalinker (Stratagene, La Jolla, CA). After incubation, the filters were washed as described above, exposed to Kodak XAR-2 film, and the activity quantified by densitometry. Glyceraldehyde phosphate dehydrogenase was used as a control in all experiments. In some experiments glyceraldehyde phosphate dehydrogenase was compared with actin and similar results were obtained.

RESULTS

To characterize the response of SCC-12F cells to TCDD under the culture conditions used for this study, we examined several parameters of growth and differentiation. Treatment of SCC-12F cultures with TCDD resulted in an increase in the state of differentiation as judged by: 1) a reduction, relative to control, in total cell number (Fig. 1A); 2) an increase in the number of cells competent to form cross-linked envelopes (Fig. 1B); and 3) an increase in involucrin mRNA levels (data not shown). Colony-forming efficiency was not significantly altered by TCDD (Fig. 1C). These results suggest that TCDD inhibited [3H]uridine incorporation by 90-95% (data not shown).
are in agreement with the reported actions of TCDD on normal and transformed human keratinocytes (4, 6, 33) and on human bronchial epithelial cells (34) and confirm that under the conditions used in this study, SCC-12F cultures respond to TCDD with a reduction in cell growth and an increase in differentiation.

The level of TGF-α mRNA is reported to be increased 96 h after treatment of confluent serum-free cultures of human keratinocytes and SCC-12F cells with TCDD (27). We have characterized the time course for changes in mRNA expression of TGF-α and two other transforming growth factors, TGF-β1 and TGF-β2 (Fig. 2). Expression of CYP1A1, a gene under direct transcriptional control by TCDD (35), was monitored as a positive control. An increase in CYP1A1 mRNA was detected at 6 h, the earliest time point examined, following addition of 10 nM TCDD to the cultures. CYP1A1 expression in TCDD-treated SCC-12F cells peaked by 24 h and remained elevated for 48 h, the latest time point examined. Significant increases in TGF-α expression were detectable 12 h after addition of TCDD. By 24 h, TGF-α mRNA levels in TCDD-treated cultures was increased 2-fold above control (2.35 ± 0.07; based on the results from 5 separate experiments). Increased levels of TGF-α mRNA in TCDD-treated cultures were maintained for 48 h. In contrast, the levels of the four characteristic TGF-β mRNA transcripts (24, 29) were decreased in TCDD-treated cultures relative to control values. By 24 h, TGF-β2 mRNA levels in TCDD-treated cultures were reduced 3-fold (0.29 ± 0.06 of control levels; based on results from 4 separate experiments). TGF-β2 expression remained depressed in TCDD-treated cells from 24 to 48 h. No statistically significant changes were observed in the levels of TGF-β1 mRNA.

Treatment of cells with the protein synthesis inhibitor, cycloheximide (10 μg/ml), for 12 h either in the presence or absence of TCDD resulted in substantial increases in the mRNAs for TGF-α and TGF-β2, relative to both control and TCDD-treated cultures (Fig. 2C). No changes in either TGF-α (0.99 ± a range of 0.28; based on the results of two separate experiments) or TGF-β2 mRNA levels (0.88 ± a range of 0.12; based on the results of two separate experiments) were observed when cells treated with TCDD plus cycloheximide were compared with cultures treated with cycloheximide alone. Unlike CYP1A1 mRNA, which is superinduced in the presence of cycloheximide (12, 44), TCDD-specific alterations in TGF-α and TGF-β2 mRNA levels are not observed in the presence of cycloheximide.

TCDD-induced changes in TGF-α and TGF-β2 mRNA levels were concentration dependent (Fig. 3). The concentration dependence for these growth factors was similar to that for the induction of CYP1A1 and was comparable to the induction of the TGF-α peptide in cultures of human epidermal cells and the SCC-12F cell line (27). The active TCDD congener, 2,3,7,8-tetrachlorodibenzo-p-dioxin, altered TGF-α and TGF-β2 to a similar degree as TCDD, while the inactive congener, 2,7-dichlorodibenzo-p-dioxin, did not alter TGF-α nor TGF-β2 gene expression (data not shown). The observed concentration dependence (Fig. 3) and stereospecificity support the involvement of the Ah receptor, previously shown to be present in these cells (36).

Nuclear run-off analysis at 90 min, 12 h and 24 h following TCDD treatment was performed to determine whether changes in TGF-α and TGF-β2 expression occurred at the level of mRNA transcription. In addition, cultures were treated with TCDD plus cycloheximide (10 μg/ml) or cycloheximide alone to evaluate the role of protein synthesis in the actions of TCDD on gene transcription. Results were compared to CYP1A1 as a positive control for transcriptional induction by TCDD. Transcription of CYP1A1 was induced approximately 2-fold 90 min following addition of TCDD (Fig. 4; Table I). By 24 h, the relative rate of CYP1A1 transcription was approximately 3.5-fold above control levels. In agreement with earlier reports (12, 44), the rate of CYP1A1 transcription was superinduced in the presence of TCDD plus cycloheximide. The rate of transcription of TGF-α was not significantly different in treated versus control cultures at any of the time points examined, either in the presence or absence of cyclo-
FIG. 3. Concentration dependence for changes in TGF-α and TGF-β2 expression. Cells were plated and treated as described in the legend to Fig. 1 for 24 h with 0.1% Me2SO as a control or TCDD at the concentrations shown above. RNA was extracted for Northern analysis as described under “Experimental Procedures.” GAPDH, glyceraldehyde phosphate dehydrogenase.

FIG. 4. Effect of TCDD on the rate of TGF-α and TGF-β2 transcription. Cells were plated and treated as described in the legend to Fig. 1 with 0.1% Me2SO (C) as a control or TCDD (T). Nuclei were collected at 24 h and nuclear run-off analysis was performed as described under “Experimental Procedures.” Actin and glyceraldehyde phosphate dehydrogenase (GAPDH) were used as loading controls. CYP1A1 was used as a positive control for the induction of transcription by TCDD. Plasmid DNA was used as a control for nonspecific binding.

| Gene       | 90 min | 12 h | 12 h + CH | 24 h  |
|------------|--------|------|----------|-------|
| CYP1A1     | 1.91 ± 0.13a | 5.21 ± 1.31a | 7.45 ± 2.10a | 3.65 ± 0.98a |
| TGF-α      | 1.27 ± 0.25a | 1.12 ± 0.15a | 1.17 ± 0.55a | 0.87 ± 0.10a |
| TGF-β1     | 0.98 ± 0.27a | 0.71 ± 0.14a | 1.24 ± 0.46a | 0.82 ± 0.09a |
| TGF-β2     | 1.05 ± 0.07a | 0.50 ± 0.16a | 0.87 ± 0.08a | 0.32 ± 0.12a |

*Values significantly different from control (p < 0.05) as determined by paired t-test.

heximide. The rate of transcription of TGF-β2 was not changed at 90 min, but was significantly reduced in the TCDD-treated cultures at 12 and 24 h. This 70% decrease in TGF-β2 transcription rate corresponds with the 70% decrease in TGF-β2 mRNA levels (Fig. 2), indicating that the decrease in TGF-β2 mRNA levels following treatment with TCDD could be directly accounted for by the decrease in TGF-β2 gene transcription. The rate of TGF-β2 transcription was not altered in cells treated with TCDD plus cycloheximide compared to cultures treated with cycloheximide alone, indicating that protein synthesis is probably required for this effect on TGF-β2.

Since no changes were detected in rate of transcription, increases in TGF-α mRNA following treatment with TCDD must occur post-transcriptionally. Studies performed to analyze the stability of TGF-α mRNA in the presence of actinomycin D showed a two-component decay curve in control and treated cultures relative to glyceraldehyde phosphate dehydrogenase mRNA (Fig. 5). Approximately 50% of the TGF-α mRNA in the absence of TCDD decayed in 3 h revealing a subpopulation of TGF-α mRNA relatively resistant to degradation. Treatment with TCDD caused an approximately 2-fold increase in the subpopulation of TGF-α mRNA resistant to degradation. The two-component decay curve was reproducible as judged by the results of two separate experiments within the same time period shown in Fig. 5. A two-component decay curve has been described previously for human growth hormone and CYP1A1 mRNA (45, 46). We must interpret the actinomycin D data with caution, since actinomycin D may modify mRNA half-lives indirectly, presumably by their effects on labile proteins or RNAs (47). Control experiments, however, showed no effects of TCDD plus actinomycin D on the decay of either actin (Fig. 5) or TGF-β2 (data not shown) mRNAs relative to SCC-12F cells treated with actinomycin D alone. These results suggest that the 2-fold increase in the resistant subpopulation of TGF-α mRNA that occurs following treatment with TCDD may be due in part to a stabilization of this TGF-α mRNA. The mechanisms involved in this stabilization of the TGF-α mRNA are not clear at this time.

**DISCUSSION**

TCDD is postulated to elicit its effects on keratinocyte growth and differentiation by altering expression of critical growth regulatory genes (1, 2). Recently, it was reported that treatment of cultured human keratinocytes with TCDD resulted in an increase in TGF-α expression (27). In this study...
we confirm the induction of TGF-α mRNA by TCDD in cultured human keratinocytes and show that the increase in TGF-α mRNA is accompanied by a reduction in the expression of TGF-β. Accompanying this increase in TGF-α mRNA is an increase in the levels of TGF-α protein measurable in the medium from TCDD-treated cultures (27). TGF-α is similar to EGF in its ability to bind, activate, and down-regulate the EGF receptor (14, 21). It has been proposed that the decrease in EGF receptor binding that occurs following TCDD treatment is due to the overexpression of TGF-α protein induced by TCDD (27).

TCDD causes an increase in TGF-α mRNA levels as a result of a post-transcriptional stabilization of the TGF-α message (Fig. 5). TCDD has also been shown to regulate the tissue-specific expression of CYP1A1 and CYP1A2, in part, through a post-transcriptional process (37, 38). Thus, post-transcriptional control may represent an important pathway by which TCDD regulates gene expression.

In contrast to TGF-α, the decrease in the steady state level of TGF-β mRNA following TCDD treatment is due to a decrease in the rate of TGF-β gene transcription. Transcriptional down-regulation is specific for TGF-β, since transcription of TGF-β1 is not affected by TCDD. Although the observed changes in TGF-β2 mRNA levels involve direct transcriptional regulation of the TGF-β2 gene, this may represent a secondary response, not necessarily regulated directly by the TCDD-Ah receptor complex, since changes in the rate of transcription of TGF-β2 occur later (after 90 min) than with CYP1A1 and do not occur in the presence of cycloheximide (Table 1). TGF-β1 and TGF-β2 are closely related polypeptides encoded by separate genes and regulated independently (17, 23). For the most part, the biological activities of TGF-β1 and TGF-β2 appear to be similar. However, TGF-β1 and TGF-β2 show some differences in their expression and activity. TGF-β1 mRNA is detected in most tissues and tumor cells investigated, while the expression of TGF-β2 is more confined to specific cell types (39, 40). In addition, TGF-β1 and TGF-β2 differentially regulate hematopoietic cell differentiation, inhibition of endothelial cell proliferation, and mesoderm induction (41-43). Furthermore, TGF-β1 and TGF-β2 are differentially expressed during the induction of terminal differentiation of cultured murine keratinocytes by calcium (24). Thus, the regulated expression of TGF-β1 and TGF-β2 is important in the normal growth and differentiation process and the differential regulation of TGF-β1 and TGF-β2 expression may be of significance in the cellular response to TCDD.

In this study we have demonstrated that TCDD induces TGF-α expression via a post-transcriptional mechanism and reduces expression of TGF-β2 at the level of gene transcription. However, regulation of TGF-β2 expression does not appear to be under direct transcriptional control of the Ah receptor. Thus, regulation of gene expression by TCDD results in differential changes in mRNA levels for specific genes and can occur by multiple mechanisms. These mechanisms include, direct transcriptional control (11, 12, 44), transcriptional control mediated as a secondary response, and post-transcriptional stabilization of mRNA.


cite{S. C. Maness and K. W. Gaido, unpublished data.}

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