Interaction between the Aryl Hydrocarbon Receptor and Retinoic Acid Pathways Increases Matrix Metalloproteinase-1 Expression in Keratinocytes*

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Exposure to the environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) results in a variety of pathological lesions in humans via activation of the aryl hydrocarbon receptor (AhR) pathway. It has become apparent that this pathway interacts with a variety of signaling pathways that are believed to be involved in mediating TCDD/AhR biological effects. Our hypothesis is that TCDD mediates these pathological lesions by directly altering the expression of genes involved in matrix deposition and remodeling and that the retinoic acid signaling pathway is involved in modulating TCDD-induced effects. Therefore, we examined the effect of TCDD and all-trans retinoic acid (atRA) on the expression of matrix metalloproteinase-1 (MMP-1, interstitial collagenase), one of the proteolytic enzymes that degrade type I collagen, in normal human keratinocytes. The data show that TCDD exposure results in increased MMP-1 expression in keratinocytes that is further enhanced by cotreatment with all-trans retinoic acid. TCDD-induced expression of MMP-1 appears to be mediated through two AP-1 elements in the proximal promoter of the MMP-1 gene. However, retinoic acid-mediated induction of keratinocyte MMP-1 is a result of both promoter activation and increased mRNA stability. These findings are the first to demonstrate TCDD-induced expression of MMP-1 and to demonstrate interactions between the TCDD/AhR and retinoic acid pathways on MMP-1 expression.

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that degrade the protein components of the extracellular matrix. MMP expression is integral to processes in skin remodeling and inappropriate expression and activity of MMPs is associated with a variety of pathologies such as rheumatoid arthritis and tumor metastasis (4–6). Regulation of MMPs occurs primarily at the levels of transcription and activity (7). Investigation into MMP gene expression have identified several cis- and trans-acting factors that are involved in MMP-1 transcriptional activation, including several AP-1 sites and PEA-3 elements, that contribute to MMP-1 gene expression (8, 9).

The primary mechanism of TCDD-induced changes in gene expression is through activation of the aryl hydrocarbon receptor (AhR/aryl hydrocarbon receptor nuclear translocator (Arnt) transcription pathway. AhR and its dimerization partner, Arnt, are members of the basic helix-loop-helix PAS (bHLH-PAS) domain family of transcription factors. Proteins in this family have diverse biological roles ranging from regulation of development, hypoxia signaling, and circadian rhythms (reviewed in Ref. 10). AhR resides in the cytoplasm in an inactive complex with accessory proteins, including two HSP90 molecules, a chaperone p23, and an immunophilin-like protein, ARA9 (XAP2, AIP) (11, 12). Binding of TCDD to AhR causes AhR to dissociate from the HSP90/p23/ARA9 complex and to translocate into the nucleus where it dimerizes with Arnt (13). The AhR/Arnt heterodimer functions as a transcriptional activator by binding to specific DNA sequences in the 5′-regions of AhR-responsive genes termed xenobiotic response elements (XRE, 5′-GCCTG-3′) (14, 15). Studies of AhR/Arnt and TCDD have focused on heterodimer ability to activate transcription of xenobiotic-metabolizing genes including members of the cytochrome p450 (CYP450) family of monooxygenase enzymes (14, 16), as well as genes unrelated to xenobiotic metabolism. These include genes encoding proteins involved in growth control, cytokines, nuclear transcription factors, and regulators of extracellular matrix proteolysis (17, 18).

Lesions resulting from TCDD exposure have been reported to arise from interactions with the retinoic acid pathway. For example, in a mouse model of cleft palate formation, exposure to RA and TCDD together result in 100% cleft palate formation at lower concentrations than are required when RA or TCDD is administered separately (21). Retinoids and vitamin A analogues are powerful regulators of cell growth and differentiation and are widely used in the prevention and treatment of a variety of cancers in humans (19). The retinoic acid (RA) signaling pathway plays a pivotal role by modulating MMP expression during these tissue-remodeling events (9, 20).

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1 The abbreviations used are: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; MMP, matrix metalloproteinase; AhR, aryl hydrocarbon receptor; Arnt, aryl hydrocarbon receptor nuclear translocator; XRE, xenobiotic response elements; RA, retinoic acid; NHK, normal human keratinocytes; TPA, 12-O-tetradecanoyl phorbol-13-acetate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcriptase; EMSA, electrophoretic mobility shift assay; atRA, all-trans retinoic acid; DRB, 5,6-dichlorobenzimidazole riboside; CHX, cycloheximide.

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tors, the retinoic acid receptors (RARs) and retinoic-x receptors (RXRs) (reviewed in Refs. 22 and 23) or through interference with other transcription factors such as AP-1--binding proteins (20, 24). MMPs are important regulators of matrix remodeling and are integral to many physiological processes, therefore, investigating the role of TCDD and the AhR/Arnt pathway in MMP activation provides a link between TCDD exposure and its pathological effects. Here we present data that show this pathway interacts with the retinoic acid signaling pathway to alter the expression of MMP-1.

MATERIALS AND METHODS
Human Keratinocyte Culture—Normal human keratinocytes (NHKs) were purchased from Cascade Biological. NHKs were incubated at 37 °C, in a humidified, 5% CO2 incubator in Media 154 supplemented with human keratinocyte growth serum (HKGS) and FSA (Cascade Biological). For experiments, confluent NHK cultures were washed three times in Ca2+/Mg2+-free phosphate-buffered saline and cultured in serum- and additive-free media containing vehicle control (MeSO) or the indicated treatment. Treatments were performed at the following concentrations: TCDD (10−3 M, UltraScientific), all-trans retinoic acid (10−6 M, Sigma), 12-O-tetradecanoylphorbol-13-acetate (TPA) (10−5 M, Sigma) α-naphthoflavone (10−6 M; Sigma), cycloheximide (CHX) (25 μg/ml; Sigma), 5,6-dichlorobenzimidazole riboside (75 μM; Sigma).

Northern Blot Analysis—Poly(A)+ RNA was isolated from confluent cultures of keratinocytes as previously described (25). Poly(A)+ RNA (3 μg) was electrophoresed in a 1.2% agarose gel containing formaldehyde and transferred onto a Immobilon NY membrane (Millipore). The blot was UV cross-linked and hybridized to 32P random primer-labeled probes. The cDNA probes used were specific to human MMP-1, PAI-2, CYP1B1, and CYP1A1. Blots were also hybridized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. Blots were exposed on phosphorimaging screens, and scanned using a PhosphorImager (Molecular Dynamics). Band intensities were analyzed using the ImageQuant 5.2 software (Molecular Dynamics).

Real Time RT-PCR—Total RNA was isolated using TRizol reagent (Invitrogen). RNA samples were reverse-transcribed using oligo(dT) primers from the SuperScript first strand synthesis system for RT-PCR (Invitrogen) per manufacturer's instructions. Primer sets for quantitative PCR are shown in Table I. Platinum quantitative PCR SuperMix-UDG (Invitrogen) was used for amplification in combination with Super Green I (Molecular Probes) for fluorescence detection. Fluorescent PCR products were detected using a Bio-Rad iCycler iQ real-time PCR detection system (Bio-Rad). Data were quantified using a standard curve generated using a vector (pCRII-TOPO, Invitrogen) containing the GAPDH promoter sequence cloned into the pGL3 vector (Promega). The GAPDH product at concentrations ranging from 1 ng to 1 pg and normalized to GAPDH to control for total RNA concentration. Melting curve and additional and normalized to GAPDH for each sample after amplification for determination of primer dimers and product amplification was verified using acrylamide gel electrophoresis.

Luciferase Constructs and Mutational Analysis—MMP-1 promoter constructs (4.4 kb to 500 bp) cloned into the pGL3-Basic luciferase vector (Promega) were the kind gift of Dr. C. Brinckerhoff. The alterations in the −70 and −180 AP-1 elements were performed using PCR mega-priming mutagenesis (26). Briefly, oligonucleotides were designed to amplify 553 bp between the −70 AP-1 or −180 AP-1 sites and the luciferase coding sequence. The oligonucleotide to the AP-1 sites contained a single base alteration in the AP-1 sequence (−70 bp: 5'-TCTTCTTAAGACGAGLTCAGAAG-3', −180 bp: 5'-TCCCTTTTGAGAAGGTAAGTCTT-3'). The products from these amplifications were cloned using Kpn1 and HindIII and cloned into the pGL3 vector for use in luciferase assays. The double mutant construct was made by performing the mutagenesis as described above using the 500 72m-pGL3 construct as template.

Transient Transfection and Luciferase Assays—NHKs were transfected with the pGL3 constructs using luciferase TransIT (Mirus) in serum- and additive-free medium (1 μg of DNA, 6 μl of TransIT). NHKs were co-transfected with the Renilla luciferase vector pRL-TK (Promega) at a ratio of 1:100 pRL-TK:pGL3 MMP-1 constructs to control for variations in transfection efficiency. NHKs were plated at a density of 2 × 105 cells/well on a 6-well plate. Twenty-four hours post-plating, cells were transfected, and the following day the cells were treated with vehicle (MeSO), TCDD, atRA, or atRA + TCDD. Cellular lysates were harvested 24 h following treatment using the dual luciferase kit (Promega), and luciferase activities determined using a Dynex Multiplate Luminometer. Protein concentrations of the lysates were determined using a modified Lowry assay (Bio-Rad). Luciferase activities were normalized to transfection efficiency using the phRL values, and to protein content. Treatments and transfections were performed in triplicate and statistical analysis done using SigmaPlot for Windows statistical analysis program (SPSS, Inc).

RESULTS
Northern Blot Analysis of MMP-1 and CYP1A1 Expression—To determine the effect of TCDD and all-trans retinoic acid (atRA) treatment on MMP-1 expression in NHK, Northern blots of poly(A)+ RNA, isolated from NHK cultures exposed to TCDD (10−3 μM), atRA (10−5 μM), atRA + TCDD or vehicle control (MeSO), were hybridized with probes to human MMP-1 as well as probes for three known AhR-responsive genes (CYP1A1, CYP1B1, and PAI-2) (Fig. 1). Exposure to TCDD increased levels of MMP-1 mRNA, in addition to the three AhR responsive genes (Fig. 1). All-trans RA alone had little effect on MMP-1 expression, and slightly inhibited RNA expression of PAI-2, CYP1A1, and CYP1B1 expression (Fig. 1, A and B). Co-treatment with atRA and TCDD resulted in further activation of MMP-1 and PAI-2 mRNA versus TCDD alone. However, CYP1A1 mRNA levels decreased by ~60% following co-treatment with atRA and TCDD. CYP1B1 steady state mRNA levels were only slightly reduced by co-treatment with TCDD and atRA in comparison with TCDD-induced levels (Fig. 1, A and B). These data demonstrate that TCDD and atRA pathways interact to modulate gene expression, and that this interaction inhibits the expression of CYP1A1, while activating the expression of MMP-1 and PAI-2.

To determine whether induction of MMP-1 mRNA by TCDD or atRA required de novo protein synthesis, NHK cultures were treated in the presence of the protein synthesis inhibitor CHX. A shorter treatment time was used (6 h) for these experiments in comparison to Fig. 1A, due to the toxicity of CHX. Co-treatment with CHX completely abolished TCDD-induced MMP-1 mRNA synthesis (Fig. 1C). Further, activation of MMP-1 mRNA expression by atRA + TCDD was also reduced to background. Therefore, TCDD and TCDD + atRA induction of MMP-1 mRNA requires protein synthesis.

To examine the involvement of the AhR pathway in TCDD and atRA activation of MMP-1 and PAI-2 expression, NHK cultures were treated in the presence of the AhR-antagonist α-naphthoflavone (Fig. 2). TCDD activation of CYP1A1 and CYP1B1 was abolished by co-treatment with α-naphthoflavone, as previously reported (Ref. 29 and data not shown). Both TCDD and atRA + TCDD activation of MMP-1 and PAI-2 were inhibited by α-naphthoflavone, indicating that the AhR pathway is involved in both TCDD- and atRA + TCDD-induced MMP-1 expression (Fig. 2). Treatment with atRA alone or in the presence of α-naphthoflavone did not appear to affect MMP-1 or PAI-2 expression (data not shown).
Retinoic Acid Co-treatment Inhibits Binding to the CYP1A1 XRE—The data presented in Fig. 1 demonstrate that TCDD-induced expression of CYP1A1 and CYP1B1 is reduced by co-treatment with atRA. Data from other laboratories suggest that atRA exposure interferes with AhR binding (30, 31). Therefore, we used mobility shift assays to determine whether atRA treatment inhibits binding to a CYP1A1 XRE sequence. Nuclear extracts were harvested from NHKs treated as above with or without co-treatment with CHX. RNA was isolated 6 h following treatment. Membranes were hybridized with probes to MMP-1 and GAPDH, exposed on a phosphorimaging screen and scanned using a PhosphorImager. Data shown are representative of three separate experiments.

FIG. 1. TCDD activation of MMP-1 and PAI-2 is further induced by atRA treatment. A, Northern blot analysis was performed on poly(A)+ RNA isolated from NHKs in serum-free media alone (Me2SO) (C) or with TCDD (10⁻⁸ M) (T), atRA (10⁻⁶ M) (R), or TCDD + atRA (TR). RNA was harvested 24 h following treatment. Membranes were probed with 32P-labeled cDNA probes specific to MMP-1, PAI-2, CYP1A1, CYP1B1 as well as GAPDH for analysis of loading. Hybridized blots were exposed to a phosphorimaging screen and scanned on a PhosphorImager. B, quantification was performed using ImageQuant software, and data plotted as fold of control (vehicle (Me2SO)) cultures. Data shown are representative of three separate experiments. C, Northern blot analysis of poly(A)+ RNA isolated from NHKs treated as above with or without co-treatment with CHX. RNA was isolated 6 h following treatment. Membranes were hybridized with probes to MMP-1 and GAPDH, exposed on a phosphorimaging screen and scanned using a PhosphorImager. Data shown are representative of three separate experiments.

FIG. 2. α-Naphthoflavone diminishes TCDD and atRA + TCDD induced expression of MMP-1 and PAI-2 mRNA in NHKs. Poly(A)+ RNA isolated from NHKs in serum-free media containing vehicle (Me2SO) or with TCDD (10⁻⁸ M) or TCDD + atRA. α-Naphthoflavone (10⁻⁶ M) was added to the cultures to block gene activation through the AhR pathway. RNA was harvested 6 h following treatment. Membranes were probed with 32P-labeled cDNA probes specific to MMP-1 and PAI-2 as well as GAPDH probe to determine loading. Hybridized blots were exposed to a phosphorimaging screen and scanned on a PhosphorImager. Quantification of expression was performed using ImageQuant software, and data are plotted as change relative to control (vehicle (Me2SO)).

FIG. 3. atRA abolishes TCDD-induced binding to the CYP1A1 XRE sequence. Nuclear extracts were isolated from NHK cultures exposed to serum-free media containing vehicle (Me2SO) (C) or media containing TCDD (T) or atRA + TCDD (TR) for 1.5 h. 5 μg of nuclear extract proteins were used in each binding reactions with a 32P-labeled probe to the CYP1A1 XRE sequence. 100-fold excess of unlabeled XRE oligonucleotide or unlabeled oligonucleotide containing the CYP1A1 sequence containing an alteration in the XRE sequence that eliminates AhR/Arnt binding (5′-GGGCGTCAG-3′ (28)) was used to compete binding. Reactions were electrophoresed on a 7% acrylamide gel. Arrow denotes XRE binding complex.
treated NHKs (Fig. 3). No binding was observed in reactions containing extracts from atRA treated NHKs (data not shown). This suggests that the reduction of CYP1A1 mRNA in atRA + TCDD treated cultures may be mediated through interference with binding to the XREs in the CYP1A1 promoter.

Expression of AhR and RAR/RXR Receptors Following TCDD or atRA Exposure—A potential mechanism mediating interactions between the atRA and AhR pathways is through alteration of receptor expression. The effect of TCDD, atRA, and atRA + TCDD on the expression of AhR and Arnt was determined using quantitative RT-PCR. NHKs were treated in serum-free media containing vehicle (MeSO) or TCDD or atRA + TCDD. Total RNA was isolated 6 h following exposure. Primers sets used to amplify AhR, Arnt, and CYP1A1 are shown in Table I.

Consistent with the data from the Northern blot analysis, results from the RT-PCR show that CYP1A1 activates CYP1A1 expression and co-treatment with atRA + TCDD results in a reduction in TCDD-induced CYP1A1 expression (Fig. 4A). Both TCDD and atRA + TCDD exposure reduce AhR and Arnt expression by -50%. However, atRA alone does not affect AhR or Arnt expression. These data suggest that the expression levels of AhR and Arnt observed in the co-treatment with atRA + TCDD are reflective of the action of TCDD alone, and that atRA does not affect AhR or Arnt mRNA levels.

To investigate the effect of TCDD on the expression of retinoid receptors, primer sets specific for RARγ and RXRα, the predominant RAR and RXR subtypes found in the epidermis (32), were used for quantitative PCR (Table I). NHK were treated in serum-free media containing vehicle (MeSO) or TCDD. Total RNA was isolated at 6, 12, and 24 h following exposure. Overall expression of RARγ was higher than that of RXRα (data not shown). We found that treatment of NHKs with TCDD results in an increase in both RARγ (4-fold) and RXRα (3-fold) mRNA messages 6-h post-treatment (Fig. 4B). This increase in RXRα mRNA was maintained up to the 24-h time point; however, message levels for RARγ returned to untreated levels or below by 12 h. These data demonstrate induction of both RARγ and RXRα mRNA in TCDD-treated keratinocytes.

TCDD Activates Expression through the 5′-Flanking Region of the MMP-1 Gene—To investigate the cis-acting elements involved in TCDD- and atRA + TCDD-induced expression of MMP-1, we performed transient transfection assays. The promoter of MMP-1 contains multiple elements involved in transcriptional activation by cytokines and the tumor promoter TPA (7). Using a transcription factor data base (MatInspector) we identified two potential XREs in the MMP-1 promoter at -1380 and -1478 bp (33). In order to determine whether these elements are involved in mediating TCDD-induced expression of MMP-1 expression, NHKs were transfected with reporter constructs containing the luciferase gene (pGL3) under transcriptional control of various lengths of the MMP-1 5′- regulatory region (Fig. 5A). Three constructs were used in these experiments: (i) the full-length region (4.4 kb), which contains both potential XREs (-1478 bp, -1380 bp) and the proximal AP-1 sites (-70 bp, -180 bp); (ii) a 1.5-kb promoter construct, containing the XREs and AP-1 sites but lacking -3 kb of 5′-upstream sequence; and (iii) 0.5-kb promoter construct, which contains the two AP-1 sites, but lack the two potential XREs.

NHKs were transfected with the luciferase constructs shown in Fig. 5A using TransIT keratinocyte reagent, and treated with TCDD, atRA, TCDD + atRA, or vehicle control (MeSO) (Fig. 5B). TCDD treatment resulted in activation of luciferase expression in NHK transfected with all three constructs (2.8–4-fold). This demonstrates that the two potential XREs in the distal portion of the MMP-1 5′-sequences are not necessary for the TCDD activation of MMP-1 and that the elements necessary for mediating the TCDD-induced MMP-1 expression are found in the minimal 0.5-kb fragment. All-trans RA alone did not elicit a significant increase in luciferase activity in any of the constructs (Fig. 5B). Co-treatment with TCDD + atRA resulted in a slight (TR/T, 1.4-fold) increase in luciferase activity over TCDD treatment alone in the cells transfected with the 4.4-kb construct. However, TCDD + atRA co-treatment of the cells transfected with the 1.5-kb construct resulted in a -50% reduction (p < 0.05) in TCDD-induced luciferase activity. No significant difference between TCDD alone and atRA + TCDD in luciferase activity is observed in cells transfected with the 0.5-kb fragment. These data suggest that the increase in MMP-1 from atRA + TCDD involves elements other than those required for TCDD alone.

TCDD Activation of AP-1 Binding Activates MMP-1 Expression—Data presented in Fig. 5 demonstrate that the two potential XREs are not required for TCDD-induced MMP expression. However, the smallest construct (0.5 kb) containing the two AP-1 elements was responsive to TCDD suggesting that the TCDD-induced increase in MMP expression may be mediated by TCDD activation of AP-1 binding to these elements. Therefore, mobility shift analysis was performed to determine if there was an increase in AP-1 binding of nuclear proteins isolated from TCDD-treated NHKs. Sequences from -45 to -78 bp of the human MMP-1 5′-region containing the AP-1 element at -70 (5′-TGAGTCA-3′) were used as a probe. Binding to this site resulted in two specific complexes (Fig. 6), as reported in the literature (27, 34). Further, specific binding to this AP-1 element was increased in extracts isolated from TCDD-treated NHK cultures.

To further examine the role of the two AP-1 elements in mediating TCDD-induced expression of MMP-1, we introduced sin-
Single base pair mutations in the AP-1 site at -70 (5' TTAGCTCA-3' to 5' GGAGCTCA-3'), in the AP-1 site at -180 (5' TTAATCA-3' to 5' GTAGCTCA-3'), or in both in the context of the 0.5-kb MMP-1 promoter fragment. This single base change in the AP-1 sequence has been shown to abolish Fos and Jun binding to the AP-1 site and eliminate AP-1 activity (27, 34). The luciferase constructs were transiently transfected into NHK and exposed to vehicle (Me2SO, C), TCDD (T) or TPA (P). TPA is known to
activate MMP-1 expression through the AP-1 elements in the proximal promoter region (7, 27). As observed in Fig. 5B, TCDD exposure resulted in an increase in luciferase activity for the 0.5-kb construct (Fig. 7). The mutation in the /H11002 72 bp AP-1 site (500/H11002 72) reduces constitutive expression driven by the promoter fragment, as has been reported in fibroblasts (27). However, mutation of the /H11002 72 bp element did not abolish TCDD nor TPA-induced expression from this promoter. Mutation of the /H11002 180 bp AP-1 (500/H11002 180) alone also did not eliminate TCDD nor TPA-induced luciferase expression. However, mutation of both AP-1 sites (dm500) eliminated both TCDD and TPA-induced expression. These data, along with data from Fig. 5, demonstrate that TCDD activation of MMP-1 mRNA expression is mediated, at least in part, through increased promoter activity. Further, these data show that TCDD induced MMP-1 expression requires both AP-1 elements.

Retinoic Acid Alters MMP-1 mRNA Stability—Our transfection data (Fig. 5B) suggests that co-treatment with atRA and TCDD results in a slight increase in the activity of the full-length MMP-1 promoter (4.4 kb) over TCDD alone. However, this level of increased transcription does not account for the amount expected from the Northern blot data (Fig. 1). Therefore we investigated the effect of these treatments on mRNA stability using the transcriptional inhibitor DRB (5,6-dichloro-benzimidazole riboside). NHKs were cultured in media containing vehicle (Me2SO), TCDD, atRA, or atRA /H11001 TCDD for 6 h. At this time, the treatment media was removed and replaced with media containing DRB. NHK poly(A) RNA was harvested at

Fig. 5. Effect of TCDD and atRA on MMP-1 promoter function. A, constructs containing 0.5–4.4 kb of the human MMP-1 promoter linked to the luciferase gene (pGL3) were used in transient transfection assays. AP-1 elements (at −180 and −72 bp) are depicted as boxes; PEA-3 elements are shown as ovals; two potential XREs are depicted as gray squares. B, luciferase activity from transient transfection of MMP-1-pGL3 constructs into NHKs. NHKs transfected with the 4.4, 1.5, and 0.5 MMP-1 pGL3 constructs were treated with TCDD (T), atRA (R), TCDD + atRA (TR), or vehicle control (Me2SO) (C) in serum-free media. Cellular lysates were harvested 24 h following treatment. Luciferase expression was assayed using the dual luciferase kit and detected on a Dynex plate reader luminometer. Treatment sets were performed in triplicate. Data were normalized to transfection efficiency by co-transfection with a Renilla luciferase control vector (phRL-TK) and to total protein content of the lysate. Error bars denote S.D. (Statistical significance was determined by the paired Student’s t test. *, p < 0.05 (C versus T or R/T); & , p < 0.05 (T versus R/T); #, p < 0.1 T versus R/T).
3, 6, and 12 h post-DRB addition. MMP-1 mRNA levels from untreated cells decreased by 40% after 3 h of DRB treatment (Fig. 8), similar to reports using other cells (35). MMP-1 mRNA levels from NHKs cultured in media containing atRA or both atRA + TCDD did not decrease during the time points tested. These results suggest that the elevated level of MMP-1 mRNA observed in atRA + TCDD cultures shown in Fig. 1 appears to result from an increase in MMP-1 mRNA stability. Accordingly, both induced expression of MMP-1 mRNA by TCDD and increased stability following atRA exposure contribute to the increased mRNA levels observed in Northern blot analysis.

**DISCUSSION**

In this study we show that exposure of NHKs to TCDD results in increased expression of MMP-1 that is mediated, at least in part, through cis-acting elements in the 5'-regulatory sequences of the MMP-1 gene. Further, our data demonstrate an interaction between the retinoic acid and AhR signaling pathways that results in either a decrease of CYP1A1 expression or an increase in MMP-1 and PAI-2 gene expression. Mobility shift experiments suggest that the decrease in CYP1A1 expression in cells co-treated with TCDD and atRA is a result of decreased AhR/Arnt binding to the XRE. However, the increase in MMP-1 mRNA levels following co-treatment with these agents involves transcriptional activation, mRNA stability, and perhaps an increase in expression of retinoic acid receptors.

Interactions between the AhR and RA signaling pathways have been reported in other laboratories and have focused on the inhibition of specific target genes, including CYP1A1 (38). Our data confirm previous reports demonstrating that co-treatment with atRA and TCDD results in an inhibition of TCDD-induced CYP1A1 gene expression (36). The results of the mobility shift experiments indicate that the reduced CYP1A1 mRNA levels may be attributable to reduced binding of the AhR/Arnt complex to the XRE. Although it is unclear how this inhibition occurs, a potential mechanism may be through competition for coactivator/corepressor proteins. Immunoprecipitation experiments show that the RA corepressor SMRT physically associates with AhR in a breast cancer cell line (37) and that the RA coactivator CBP/p300 interacts with the C-terminal domain of Arnt (38). Nuclear receptor corepressors are reported to function by causing deacetylation of adjacent chromatin structures resulting in transcriptional silencing (39–41). Consistent with this view, it has been shown that in fibroblasts Arnt interacts with an unknown factor that inhibits CYP1A1 activation, and treatment of these cells with an acetylase inhibitor releases fibroblasts from this inhibition (42).

Another potential interaction between the AhR and RA signaling pathways is through expression of receptors. The results of experiments reported here show no alteration of AhR or Arnt expression following atRA exposure. However, a decrease in both AhR and Arnt mRNA levels was observed following TCDD treatment. Studies using the murine AhR promoter demonstrate that treatment of a murine epidermal cell line with either TCDD or atRA results in reduced AhR promoter activity (43). Therefore, our observed decrease in AhR expression following TCDD exposure may reflect a reduction in AhR promoter activity. However, we observed no decrease in AhR gene expression following atRA treatment. This difference may be a consequence of species-specific differences between the human and murine AhR promoter activity or from a difference in atRA responsiveness of the human versus the murine cell line tested.

In support of this idea is the fact that the murine cell line (JBG-C1 41-5a) used in the reported studies is highly responsive to retinoic acid (43).

The effect of TCDD on RAR and RXR gene expression appears to be receptor- and cell type-dependent. For example, in SCC12Y cells TCDD treatment results in a decreased binding of atRA to RARα without any change in RARα gene expression (44). In contrast, TCDD-treated murine palate mesenchymal cells show a decrease in atRA-induced RARβ gene expression (31). Our results demonstrate that TCDD treatment causes an increase in RARγ and RXRα mRNA levels in NHKs. Although both genes are activated by TCDD exposure, there are differences in their activation. RARγ expression is elevated by 6 h of treatment and is maintained through 24 h, whereas RXRα expression drops to basal levels after 12 h of TCDD treatment. RARγ and RXRα compose the predominant RAR/RXR heterodimer in normal human skin (32, 45), and therefore TCDD-induced alterations in gene expression of RARγ and RXRα may influence RA-mediated gene expression.

Our finding that TCDD alters expression of MMP-1 provides a potential underlying mechanism for many of the lesions resulting from TCDD exposure. In support of our finding, MMP-1 and MMP-9 were recently identified as TCDD-target genes in human airway epithelial cells using microarray analysis (46). This suggests that TCDD-induction of MMPs may be a common mechanism underlying TCDD-induced pathologies. Indeed, many observed TCDD-induced lesions are related to disruption in matrix remodeling. Chloracne is the most consistent early lesion observed in TCDD-exposed humans (47). This hyperkeratotic condition is believed to result from alterations in keratinocyte differentiation and hyperkeratization of the follicular and the interfollicular epithelium (48). Experimental evidence from animal models demonstrates that TCDD affects the architecture of other tissues as well. In utero and lactational exposure of rats and mice to TCDD results in altered structures of a variety of organ systems, including seminal vesicle devel-
opment (1), aberrant mammary development (3), and cleft palate (2). All of these processes require extensive tissue remodeling and involve the expression and activity of MMPs (49).

Data presented in Fig. 5A demonstrate that TCDD-induced MMP-1 mRNA is mediated, in part, through increased transcription and requires the AP-1 sites in the proximal promoter. Although TCDD-induced MMP-1 expression is not mediated through direct XRE binding in the MMP-1 promoter, the data show that the induction requires AhR activity as well as de novo protein synthesis. This suggests that the effect of TCDD on MMP-1 expression may be mediated through TCDD-induced expression of Fos and Jun proteins, which in turn bind to and activate the AP-1 elements in the MMP-1 proximal promoter (Fig. 9). Data from other laboratories demonstrate that TCDD-exposure of hepatoma cells enhance expression of c-Fos, c-Jun, JunB, and JunD as well as AP-1 binding (50). This supports our finding that TCDD exposure of NHKs activates AP-1 binding and that TCDD-induced MMP-1 expression requires both de novo protein synthesis and AhR signaling. Loss of TCDD induction of the MMP-1 promoter requires removal of both AP-1

![Two AP-1 sites in the MMP-1 promoter are critical for TCDD-induction.](image)

**Fig. 7.** Two AP-1 sites in the MMP-1 promoter are critical for TCDD-induction. Luciferase activity of MMP-1-pGL3 promoter constructs transiently transfected into NHKs was analyzed. NHKs were transfected with luciferase constructs (pGL3) containing the minimal (500 bp) MMP-1 fragment or constructs containing a single base pair mutation in the −72 AP-1 site. Transfected cultures were treated with TCDD (10−8 M) (T), TPA (10−8 M) (P), or vehicle control (Me2SO) (C) in serum-free media. Cellular lysates were harvested 24 h following treatment. Luciferase expression was analyzed using the dual luciferase kit and detected on a Dynex plate reader luminometer. Treatment sets were performed in triplicate. Data were normalized to transfection efficiency by co-transfection with a Renilla luciferase control vector (phRL-TK) and to total protein content of the lysate. Data are presented as fold of control (vehicle (Me2SO)) and are representative of three separate experiments.

![MMP-1 mRNA is stabilized following atRA treatment of NHKs.](image)

**Fig. 8.** MMP-1 mRNA is stabilized following atRA treatment of NHKs. NHKs were cultured in serum-free media alone (Me2SO) or containing TCDD (10−8 M), atRA (10−8 M) or TCDD + atRA. Six hours later, treatment media was removed and replaced with media containing the transcriptional inhibitor DRB (75 μM). Poly(A)+ RNA was isolated prior to the addition of DRB and at 3, 6, and 12 h following DRB exposure. Membranes were probed with 32P-labeled cDNA probes specific to MMP-1 and GAPDH probes. Hybridized blots were exposed to a phosphorimaging screen and scanned on a PhosphorImager. Quantification was performed using ImageQuant software. Data are shown as the percent (%) of 6 h MMP-1 mRNA levels prior to DRB addition and are the average of two separate experiments.
elements in the proximal promoter, suggesting that either the −72 bp or the −180 bp AP-1 element is sufficient for TCDD-induced promoter activity.

The amount of MMP-1 mRNA is increased by co-treating NHKs with atRA and TCDD. All-trans RA is an inhibitor of MMP gene expression in other cell types (9, 51); however, recent reports demonstrate atRA-induced MMP protein expression in NHKs (52). This suggests that MMP gene expression may be regulated differently by atRA in epithelia in comparison with fibroblastic cell types (52, 53). Our data support this finding and indicate that atRA may impact MMP-1 expression through both transcriptional and post-transcriptional mechanisms (Fig. 9). The transfection data show a slight but significant increase in TCDD-induced activity of the largest MMP-1 promoter fragment when cells were co-treated with TCDD and atRA. Therefore, the sequences involved in mediating the increase following TCDD and atRA treatment differ from those identified as important for TCDD induction alone. Although it is unclear at this time what sites in the distal promoter may be contributing to the atRA + TCDD induced MMP-1 promoter activity, a PEA-3 site at −3108 bp in this region has been identified as being a target for atRA (9).

The increase observed in luciferase activity driven by the MMP-1 promoter following co-treatment with TCDD and atRA was slight and did not account for the increase in mRNA levels observed in the Northern blot analysis. However, an increase in MMP-1 mRNA stability was observed in the co-treated NHKs, as well as in the atRA alone-exposed cells. Therefore, the increase in steady state MMP-1 mRNA following TCDD-atRA co-treatment results from a slight increase in transcription in addition to an increase in MMP-1 mRNA stability. Increased MMP-1 mRNA stability following atRA treatment has also been observed by other laboratories (53).

In summary, the results of experiments presented here elucidate how TCDD induces MMP-1 expression in normal human keratinocytes. Further, it is shown that co-treatment with TCDD and RA results in an additive increase in MMP-1 expression and that this interaction involves both transcriptional and post-transcriptional mechanisms. It has become apparent that the AhR pathway interacts with a variety of signaling pathways, and that these interactions are critical to TCDD/AhR biological effects. Because matrix remodeling is a key process in cell migration and tumor invasion, our results provide insight into the mechanism and function of the AhR pathway and its impact on these processes in skin. Moreover, the retinoic acid pathway is targeted for pharmacologic therapy for skin disorders, including non-melanoma skin cancer and cystic acne. Understanding how the AhR pathway impacts retinoic acid signaling in skin may contribute to the design of synthetic retinoids for therapeutic use.

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