INFLAMMATORY SIGNALING COMBINED WITH DIOXIN-MEDIATED AH RECEPTOR ACTIVATION MEDIATES SYNERGISTIC INDUCTION OF IL-6

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Introduction

TCDD has been shown to be a potent promoter of ovarian, liver, and skin tumor formation in two-stage carcinogenesis rodent models[1,2,3]. TCDD exhibits its ability to promote tumor development almost solely through continual activation of the Aryl hydrocarbon receptor (AHR). Transgenic mice expressing a constitutively-active mutant of the AHR, form liver tumors after exposure to N-nitrosodiethylamine as an initiator[4]. The precise mechanism(s) of AHR-mediated tumor promotion are largely unknown, although several theories have been proposed. One common finding is that long-term exposure to TCDD appears to increase breast cancer risk. Research in our lab has provided evidence that AHR activation in cells, in combination with inflammatory signaling, leads to synergistic increases in the production of the cytokine interleukin-6 (IL-6)[5]. Long term induction of IL-6 may potentially act as a tumor promotion event.

IL-6 is a pleiotropic cytokine "classically" involved in the acute phase response, as well as lymphocyte differentiation and proliferation following inflammatory stimuli. Additionally, IL-6 expression in tumor cells is known to elicit both pro- and anti-tumorigenic properties. Elevated expression of IL-6 in prostate cancer cells has been demonstrated to enhance androgen receptor-dependent growth[6], decreased sensitivity to chemotherapeutic drugs[7], and resistance to apoptosis[8]. Numerous clinical studies have shown that cervical cancer is associated with elevated IL-6 levels and enhanced IL-6 gene expression has been correlated with the presence of invasive cervical carcinomas[9,10,11]. However, IL-6 expression in breast cancer has been linked to both positive and negative effects on overall prognosis[12]. Low basal IL-6 levels have been observed in certain established breast cancer cell lines, such as MCF-7 cells[13]. Whether the lack of IL-6 expression enhances or represses the tumorigenic potential or ability to metastasize *in vivo* is poorly understood and is dependent on the tumor type as well as the level of tumor progression.

Materials and Methods

MCF-7, ECC-1, and BEAS-2B cells were plated and serum starved for 18 hours prior to treatment. Treatment of cells was performed by diluting IL-1β to 10 ng/ml and TCDD to 1 nM in serum-free media supplemented with 3 mg/ml BSA or 1% dextran-charcoal stripped serum. Conditioned media was created by adding PMA at 81 nM for 24 hours to undifferentiated THP-1 cells. Differentiated THP-1 cells were then washed, followed by the addition of serum-free media containing 100 ng/ml LPS, and incubated for 24 h. Gene expression levels were determined by extracting total RNA, preparing cDNA, and measuring mRNA expression levels by quantitative real-time PCR. Values shown are either raw quantities or target gene quantities normalized to levels of the housekeeping gene GAPDH. To quantify IL-6 protein expression by ELISA, media was collected from treated cells at 24, 48, and 72 h post-treatment and incubated in optical dishes pre-bound with anti-human IL-6 monoclonal antibody. Plates were then washed and incubated with biotinylated goat anti-human IL-6 antibody, washed and incubated with diluted streptavidin, then washed and finally incubated with colorimetric assay reagent. Assays were performed by reading spectrophotometric absorbance and analyzing resultant data. In certain experiments AHR mRNA levels were decreased using Dharmacon siRNA oligos transfected using the Amaxa nucleofection/electroporation system. Control or targeted siRNA was added to the sample for a final concentration of 2 μM per sample. Samples were electroporated using manufacturer's high efficiency program, and plated into 6 well dishes in complete media.

Results and Discussion

Co-treatment of MCF-7 cells with TCDD and IL-1β results in a synergistic induction of IL-6

Data collected in our laboratory show molecular and transcriptional changes that some cells undergo in response to a combination of pro-inflammatory IL-1β signaling and AHR agonist binding. As shown in figure 1, MCF-7 ER-positive breast cancer cells, when exposed to 1 nM TCDD and 10 ng/ml IL-1β, have a synergistic increase in IL-6 transcription within 2 hours of treatment. Simultaneously, the addition of IL-1β decreases CYP1A1 transcription levels in response to TCDD. Combinatorial treatment with TCDD and PMA has been shown to have a similar effect, with IL-6 transcription levels increasing in a dose-dependent manner with regard to both PMA and TCDD. In addition to TCDD, other AhR agonists including benzo[*a*]pyrene were found to induce comparable increases in IL-6 transcription.

This synergistic IL-6 induction was also detected at the protein level by ELISA assay and continued throughout a 72 hour treatment period. The finding that levels of secreted IL-6 protein are increased synergistically holds potential for both autocrine and paracrine signaling between tumor cells and their microenvironment. In line with this notion is the finding that IL-1β secreted by macrophages is sufficient to induce synergistic IL-6 transcription in the presence of TCDD without the addition of exogenous IL-1β. The implication is that physiological levels of pro-inflammatory IL-1β, in combination with low levels of TCDD exposure, may be sufficient to induce a synergistic increase in pro-tumorigenic IL-6 output.

IL-6 transcription is dependent upon the AHR signaling pathway and NF-κB

To explore the molecular mechanism by which TCDD leads to increased IL-6 transcription in the presence of IL-1β, the focus began with the canonical AHR signaling pathway and corresponding transcription factors. As shown in figure 2, loss of AHR by siRNA treatment leads to a loss of IL-6 transcriptional changes. Similarly, loss of the AHR's dimerization partner, ARNT, leads to a loss of IL-6 induction. The combination of these two experiments verifies that TCDD is acting through the typical AHR signaling pathway of intracellular dimerization with ARNT and presumably, nuclear translocation and DNA binding. Current research is being undertaken to assess the necessity of DNA binding by the AHR for this synergistic effect. The presence of an imperfect dioxin response element (DRE) sequence roughly 2 kb upstream from the IL-6 transcription start site raises the question of whether ligand-bound AHR is binding outside of the proximal promoter and affecting IL-6 transcription from a distance. We have also conducted siRNA experiments targeting various transcription factors involved in IL-6 signaling, including C/EBPβ, c-Jun, and the RelA subunit of NF-κB. Blocking RelA expression had a significant effect on the induction of IL-6. Additionally, we have found that IL-8, which has a transcriptional regulation mechanism very similar to that of IL-6, is not synergistically induced. This reinforces the notion that the event is very context-specific with regard to the promoter. ChIP assays of the IL-6 promoter also show an increase in acetylated histones upon co-treatment of IL-1β and TCDD, when compared to individual treatment or controls. This is indicative of an increase in DNA remodeling at the IL-6 promoter; a hallmark of increased gene expression.

Synergy is observed to occur in a cell type specific manner

In addition to the MCF-7 breast cancer cell line, some malignant and non-tumorigenic cell culture lines have shown similar IL-6 effects upon co-treatment with TCDD and IL-1β. Figure 3A shows the synergistic induction of IL-6 in ECC-1 endocervical carcinoma cells following similar treatment conditions. We have shown that ECC-1 cells follow the same patterns as MCF-7 cells in CYP1A1 repression and IL-6 protein increase over 72 hours. Shown in figure 3B is the response of the normal lung epithelial cell line BEAS-2B. Other cell lines that tested negative for synergistic increases in IL-6 include head and neck carcinoma cell lines and ER-negative breast cancer cell lines. These findings point to a very cell-specific setting in which IL-1β and TCDD combine for synergistic IL-6 production, but also demonstrate that the effect is not relegated solely to carcinoma cells. Future research will attempt to clarify the role of transcription factor binding site proximities within the IL-6 proximal promoter in order

to test the hypothesis that positively-regulated cell lines share similarities in DRE, NF-κB, and other sequence locations.

These findings show that, in MCF-7 ER-positive breast cancer cells, the combination of pro-inflammatory IL-1β and TCDD leads to a synergistic increase in IL-6 output. The event requires AHR activation and dimerization, and work is underway to clarify the necessity of DNA binding. While the event is NF-κB dependent, it is context-specific in that other NF-κB driven genes are not similarly synergized. Finally, other cancerous and non-cancerous cells exhibit a similar response to this combinatorial treatment, but it is by no means universal across cell cultures. Future research is aimed at elucidating the exact molecular mechanism of the synergistic induction and an understanding of the process by which AHR may prime the IL-6 promoter for increased transcription. Functional assays will also be performed in an effort to determine the physiological relevance of the increased IL-6 output. As increased IL-6 has been linked to anti-apoptotic, pro-growth, and increased invasiveness in different cancer models, functional assays will clarify whether the synergistic IL-6 output is one mechanism in which AHR functions in conjunction with inflammatory signaling as a tumor promoter.

Acknowledgements
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Figure 1

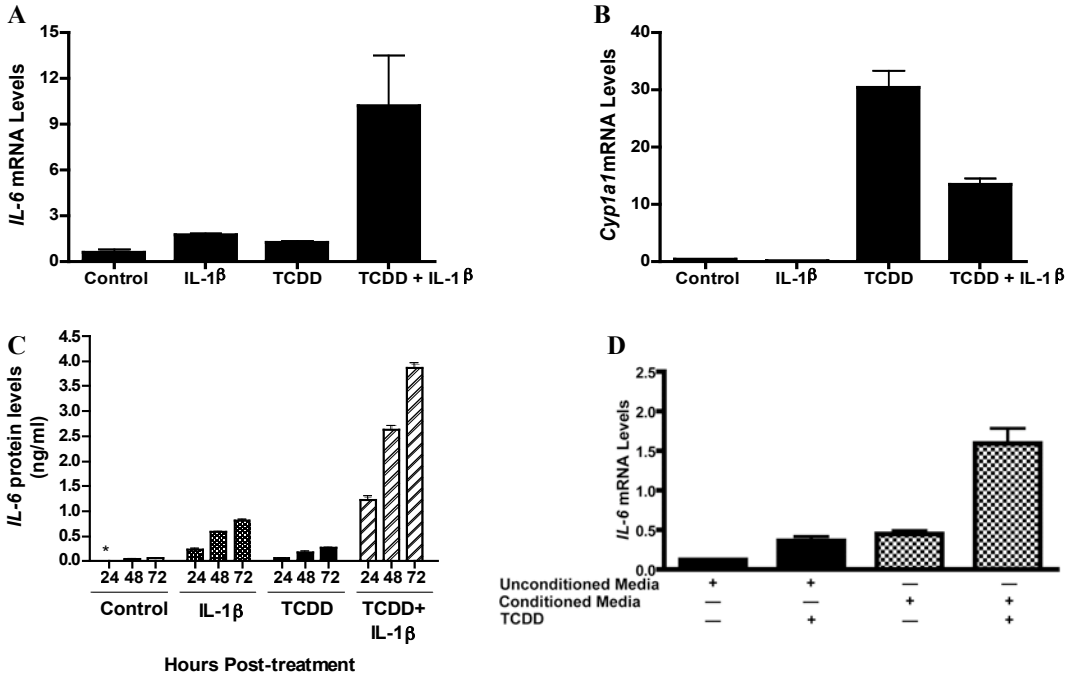

Figure 2

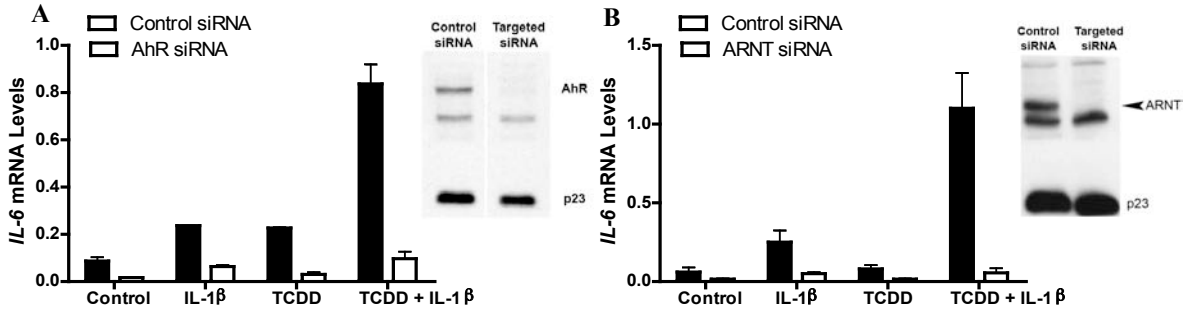

Figure 3