Immunomodulatory drugs inhibit expression of cyclooxygenase-2 from TNF-α, IL-1β, and LPS-stimulated human PBMC in a partially IL-10-dependent manner

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Abstract

Immunomodulatory drugs (IMiDs) are potent inhibitors of TNF-α and IL-1β and elevators of IL-10 production in LPS-stimulated human PBMC. They are currently in clinical trials for various diseases, including multiple myeloma, myelodysplastic syndrome, and melanoma. In the present study, we have investigated the effects of thalidomide, CC-5013 and CC-4047 on the expression of COX-2 by stimulated PBMC. Our results show that thalidomide and IMiDs inhibited the expression of COX-2 but not the COX-1 protein in LPS-TNF-α and IL-1β stimulated PBMC and shortened the half-life of COX-2 mRNA in a dose-dependent manner. They also inhibited the synthesis of prostaglandin E2 from LPS-stimulated PBMC. While anti-TNF-α or IL-1β neutralizing antibodies had no effect on COX-2 expression, anti-IL-10 neutralizing antibody elevated the expression of COX-2 mRNA, and protein from treated PBMC. These data suggest that the anti-inflammatory and anti-tumor effects of IMiDs may be due in part to elevation of IL-10 production and its subsequent inhibition of COX-2 expression.

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1. Introduction

Thalidomide has been shown to exhibit anti-angiogenic, anti-inflammatory properties via a yet unknown molecular mechanism. It has a wide spectrum of pharmacological activities against clinical conditions such as Crohn’s disease, graft versus host disease, Behcet’s disease, erythema nodosum leprosum, and several hematopoietic and solid tumors [1–10].

To obtain more potent immunomodulatory drugs than thalidomide, without the teratogenic side effects, several structural analogs of thalidomide have been synthesized and tested on LPS1-stimulated PBMC for their inhibition of TNF-α production. New analogs of thalidomide, the immunomodulatory drugs (IMiDs) are shown to be at least 50,000-fold more potent than thalidomide in inhibiting TNF-α production by LPS-stimulated monocytes. This class of analogs is also shown to inhibit IL-1β and IL-6 and enhance IL-10 production in a dose-dependent manner by LPS-stimulated human PBMC [11–14]. Furthermore, IMiDs have also been shown to inhibit angiogenesis in several in vitro and in vivo experiments. Given all of these

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Abbreviations used: TNF, tumor necrosis factor; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cells; DMSO, dimethyl sulfoxide; PGE2, prostaglandin E2; COX, cyclooxygenase.

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properties, IMiDs are currently undergoing evaluation in the treatment of various hematopoietic and solid tumors [15–18].

Cyclooxygenase (COX) is an enzyme that catalyses the transformation of arachidonic acid into prostaglandin. Three isoforms of the COX enzyme have been identified: COX-1, which is constitutively expressed; COX-2, which is induced in several cell types by pro-inflammatory stimuli such as LPS, TNF-α and IL-1β; and COX-3, the function of which has been less well characterized, but which has been shown to be the target of acetaminophen [19–22]. The induction of COX-2 expression by TNF-α and IL-1β in cancer cells was shown to be through the activation of the NF-κB pathway [23]. Several reports have shown that the anti-inflammatory cytokine IL-10 is a potent inhibitor of macrophage function, blocking synthesis of IL-1β, TNF-α, IL-6, IL-8, and COX-2 [24–26]. These studies suggested that IL-10 plays a role in the regulation of COX-2 expression from activated monocytes. Numerous studies have demonstrated that COX-2 but not COX-1 overexpression plays a major role in the pathogenesis of different types of cancers, such as head and neck, breast, lung, colon, and pancreas [27–30]. Over-expression of COX-2 has been demonstrated in different animal models of inflammation and tumors [31,32].

Recent studies also demonstrated that overexpression of COX-2 played a role in the tumor angiogenesis process [33,34]. Saha et al. [35] have shown that the anti-angiogenic small molecule SU5416 was able to inhibit the endogenous as well as the phorbol ester-induced expression of COX-2 in human lung carcinoma cells. This suggests that the ability of a small molecule to inhibit angiogenesis might be related to its inhibitory activities on the expression of the COX-2 protein. Other studies have shown that non-steroidal anti-inflammatory drugs (NSAIDs) such as celecoxib inhibit the growth of a tumor size and proliferation of tumor cells by a COX-2-dependent mechanism [36].

In the current study, we have examined the influence of IMiDs on the expression of COX-2 from stimulated monocytes. Our results indicate that IMiDs inhibited the expression of the COX-2 protein and mRNA from LPS-stimulated PBMC in a dose-dependent manner. The effects of thalidomide and IMiDs were also investigated on the expression of COX-2 by human PBMC stimulated with proinflammatory cytokines such as TNF-α and IL-β. Neutralizing antibody against IL-10 but not TNF-α or IL-1β elevated COX-2 expression in CC-5013 treated samples indicating that CC-5013-mediated IL-10 upregulation plays a role in COX-2 expression. Our results provide new information for understanding the anti-inflammatory and anti-angiogenic activities of thalidomide and the IMiDs.

2. Materials and methods

2.1. Antibodies and reagents

IMiDs and thalidomide (Celgene Corporation, Warren, NJ) were dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions. The final concentration of DMSO was kept at 0.1% for all the assays. All the antibodies for the Western blot were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cells

Peripheral blood mononuclear cells (PBMC) were isolated from leukocyte units buffy coat, (Blood Center of New Jersey) from healthy donors by Ficoll–Hypaque gradient centrifugation. The serological status of donors was performed by ELISA assay at the Blood Center of New Jersey.

2.3. Western blotting

PBMC were plated at 2.5 × 10^6 cells per ml media (as described in Section 2.2) overnight. For COX-1 and COX-2 expression: PBMC were pretreated with either thalidomide or IMiDs for 1 h and stimulated with either LPS (10 ng/ml) or TNF-α (10 ng/ml) or IL-1β (15 ng/ml). At different time points, the cells were then lysed in a RIPA buffer and generated cell lysate. Approximately, 50 μl of this mixture was loaded per lane on 10% Tris-glycine polyacrylamide gels (Novex), electrophoresed, and transferred to PVDF membranes (Novex). PVDF membranes were blocked for 1 h and incubated overnight at 4 °C with COX-1, COX-2, IL-1β, TNF-α, IL-10 or β-actin polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz CA). Membranes were then developed using the ECL Plus chemiluminescent detection system (Amersham–Pharmacia Biotech).

2.4. Northern blotting

The total RNA was prepared using the TriZol reagent according to the manufacturer’s protocol (Invitrogen). Total cellular RNA (20 μg) was electrophoresed through 1% agarose–formaldehyde denaturing gels and transferred to a positively charged nylon membrane (Ambion BrightStar-Plus). The filters were hybridized with radiolabeled COX-1, COX-2 or GAPDH. DNA probes were labeled with ^32P by the random primer method. For mRNA stability, PBMC (1.5 × 10^7 cells per treatment) were pretreated with either 0.1% DMSO or different concentrations of IMiDs for 1 h. The treated cells were then stimulated with LPS (10 ng/ml). After selected time points, actinomycin D was added to each sample at a final concentration of 10 μg/ml. The total RNA was extracted at various time points and COX
mRNAs were evaluated by using Northern blot analysis as described above. Autoradiographic signals were quantified on a Storm Phosphor Imager (Molecular Dynamics), and then normalized to GAPDH signals.

Northern blots were performed using 20 μg of total cellular RNA electrophoresed through 1% agarose–formaldehyde denaturing gels and transferred to a positively charged nylon membrane (Ambion BrightStar-Plus). The filters were hybridized with radiolabeled COX-2 or GAPDH probes using T4 polynucleotide kinase.

For COX-2 RNA stability, 3 × 10^7 cells per treatment were used in a final volume of 10 ml. The cells were treated with either DMSO (0.1%) or CC-5013 (1 μM) for 1 h followed by the addition of LPS at a final concentration of 10 ng/ml. Two hours after the addition of LPS, actinomycin D was added at a final concentration of 5 μg/ml. The treatments were harvested at either time zero, 1 or 2 h after actinomycin D.

Autoradiographic signals were quantified on a Storm Phosphor Imager (Molecular Dynamics) and then normalized to GAPDH signals.

### 2.5. COX-2 activity and PGE2 ELISA assays

The COX-2 activity assay was performed according to the procedure from Cayman Chemical Company (Ann Arbor, MI). In this assay, purified Ovine COX-1 and COX-2 enzymes were used and activity was determined using a colorimetric enzyme assay. PGE2 in medium was measured using an ELISA assay from Cayman Chemical Company. A total of 1 × 10^6 cells were plated in triplicate in serum-free media. The cells were then pretreated for 1 h with different concentrations of the compound and stimulated with PMA (1 ng/ml) for additional 3 h. Arachidonic acid (20 μM) was added to stimulated cells and incubated for additional 3 h. Supernatants were used in the ELISA assay to determine the amount of PGE2 following the manufacturer’s procedure.

### 3. Results

#### 3.1. IMiDs inhibit the expression of COX-2 protein from LPS, IL-1β and TNF-α stimulated PBMC

To assess whether IMiDs regulate COX-2, we examined the expression of COX-2 from stimulated PBMC by Western blot analysis. PBMC were pretreated with various concentrations of either CC-5013 or CC-4047 for 1 h, then stimulated with LPS, TNF-α or IL-1β for 24 h. As shown in Figs. 1A and B, unstimulated cells do not express the COX-2 protein. Upon stimulation with LPS, TNF-α or IL-1β there is a significant increase in expression of COX-2 compared with unstimulated cells. The addition of CC-5013 or CC-4047 inhibited this expression in a dose-dependent manner. COX-1, on the other hand, was constitutively expressed and there was no inhibitory effect after the addition of CC-5013 even at 10 μM when compared with LPS treated cells (Fig. 1C). The results from these experiments indicated that the inhibitory effects of IMiDs are specific for COX-2 from a stimulated monocyte population.

#### 3.2. IMiDs inhibit the expression and stability of COX-2 mRNA from LPS-stimulated PBMC

To investigate the effects of IMiDs on the expression of COX-2 mRNA, PBMC were pretreated with drugs for 1 h and then stimulated with LPS for various lengths of time (2, 4, 8, and 20 h). As shown in Fig. 2A, stimulation with LPS induced the expression of COX-2 mRNA compared with DMSO treatment. The addition of CC-5013 inhibited the expression of COX-2 mRNA at the later time points (8 and 20 h). At 20 h, the amount of COX-2 mRNA was also inhibited in LPS treated cells. Our explanation for this is that longer exposure of cells to LPS caused enhancement in the secretion of IL-10 protein which resulted in inhibition of COX-2 mRNA in both LPS and LPS plus drug treated cells.

The effects of thalidomide, CC-5013 and CC-4047 were also tested on the expression of COX-2 mRNA at 1
and 10 μM. As shown in Fig. 2B, all three agents inhibited the expression of COX-2 mRNA in a dose-dependent manner. Thalidomide was shown to be less potent than IMiDs in this experiment and other in vitro experiments (data not shown). To understand the mechanism by which CC-5013 inhibits COX-2 protein expression, we investigated the effects of the CC-5013 on the steady state levels of COX-2 mRNA from LPS stimulated PBMC in the presence or absence of actinomycin D, an RNA synthesis inhibitor. In this experiment, cells were pretreated with CC-5013 at 1 μM for 1 h and then stimulated with LPS for 4 h. After stimulation, actinomycin D was added and the total RNA was harvested at indicated time points and subjected to Northern blot analysis. As it shown in Fig. 2C, stimulation with LPS increased the COX-2 mRNA compared with the control treatment, and the addition of CC-5013 inhibited the expression. Furthermore, treatment with CC-5013 enhanced the degradation rate of COX-2 mRNA in a time-dependent manner.

3.3. Effects of IMiDs on enzymatic activity of COX-1 and COX-2

The effects of IMiDs were also investigated on the activity of both COX-1 and COX-2. For this, the effects of CC-5013 and CC-4047 were tested on purified ovine
COX-1 and COX-2 enzymes using colorimetric enzyme assays. As shown in Figs. 3A and B, the drugs did not have any effect on the activity of either COX-1 or COX-2. Aspirin was used as a positive control and was shown to inhibit the activity of both COX-1 and COX-2 in a concentration dependent manner. Etodolac and NS398 were also used as positive controls and were shown to inhibit COX-2 but not COX-1 activity. These results indicated that IMiDs inhibit the expression but not activity of the COX-2 protein.

3.4. CC-5013 increases the expression of IL-10 mRNA and protein from LPS-stimulated PBMC

Previous reports have shown that IMiDs were able to inhibit IL-1β and TNF-α production by LPS-stimulated PBMC in a dose-dependent manner [11]. In this study, we investigated the effects of CC-5013 on the expression of IL-10 mRNA. As shown in Fig. 4, LPS stimulation enhanced the expression of IL-10 mRNA in a time-dependent manner, which peaked at 8 h. The addition of CC-5013 at 1 μM enhanced IL-10 mRNA expression over LPS-stimulated cells in a time-dependent manner. To investigate the effects of CC-5013 on expression of IL-10 protein, the PBMC were pretreated with either CC-5013 or CC-4047 at 1 μM, and treated cells were then stimulated with LPS (10 ng/ml) for 48 h. The supernatants were analyzed for IL-10 production by ELISA. Data shown are representative of three similar experiments.

Fig. 3. CC-5013 and CC-4047 have no effect on activity of COX-2 in an enzymatic assay. The COX-2 activity assay was performed according to the procedure from Cayman Chemical Company (Ann Arbor, MI). In this assay purified Ovine COX-1 and COX-2 enzymes were used and activity was determined using a colorimetric enzyme assay.

Fig. 4. CC-5013 enhances the expression of IL-10 mRNA and protein in a dose-dependent manner. PBMC from healthy donors were pretreated 1 h with CC-5013 and then stimulated with LPS (10 ng/ml). Total RNA was harvested at various time points as indicated in graph and 20 μg of each sample was subjected to Northern blot analysis as described in Section 2. (A) Effects of CC-5013 on expression of IL-10 mRNA over time. (B) PBMC were pretreated with CC-5013 at 1 μM. The treated cells were then stimulated with LPS (10 ng/ml) for 48 h. The supernatants were analyzed for IL-10 production by ELISA. Data shown are representative of three similar experiments.

3.5. Neutralizing antibody to IL-10 but not IL-1β or TNF-α partially reverses the inhibitory effects of IMiDs on COX-2 expression

The anti-inflammatory cytokine IL-10 has been shown to regulate the expression of COX-2 [24]. Since IMiDs enhanced the secretion of IL-10, we investigated the potential role that IL-10 might play in the inhibition of COX-2 by IMiDs. To do this, cells were pre-treated with CC-5013 and then stimulated with LPS in the absence or presence of a neutralizing antibody to IL-10,
TNF-α or IL-1β. As shown in Fig. 5A, LPS stimulation enhanced the expression of COX-2 compared with unstimulated cells. The addition of CC-5013 significantly inhibited the expression of COX-2 compared with LPS-stimulated cells. The addition of a neutralizing antibody to IL-10 but not isotype control increased the COX-2 expression in LPS- and LPS + CC-5013-treated cells, thus confirming the inhibitory role of IL-10 in COX-2 expression. In a Northern blot analysis using a neutralizing antibody to IL-10, we also obtained similar results (Fig. 5C). Anti-IL-10 antibody increased COX-2 mRNA levels in CC-5013 treated samples. Therefore, CC-5013-induced IL-10 production plays a role in COX-2 expression, but we cannot rule out an IL-10-independent mechanism of CC-5013-mediated COX-2 inhibition.

The role of pro-inflammatory cytokines TNF-α and IL-1β in CC-5013-mediated COX-2 inhibition was also investigated. Our results indicated that neutralizing antibodies to either TNF-α or IL-1β had no significant effect on COX-2 protein levels compared to the isotype control antibody (Fig. 5B).

3.6. IMiDs inhibit the synthesis of PGE2 from LPS-stimulated PBMC in a dose-dependent manner

To assess whether the increased COX-2 synthesis was associated with an increase in the formation of PGE2, we investigated the release of PGE2 in response to LPS in the presence of arachidonic acid. As shown in Fig. 6, LPS increased the synthesis of PGE2 about sixfold over unstimulated cells in the presence of exogenous arachidonic acid. The addition of CC-5013 or CC-4047 inhibited the secretion of PGE2 in a dose-dependent manner.

4. Discussion

COX-2 is secreted by several cell populations such as fibroblasts, endothelial cells, and monocytes/macrophages in response to different stimuli [37,38]. Macrophages are known to play a major role in the innate immune defense system against pathogens and tumor cells and represent one of the main cellular sources of COX-2 expression upon exposure to different stimuli [19]. Reports by different laboratories have shown that these cells are abundant in the stroma of several tumors and are potentially significant sources of pro-inflammatory cytokines and pro-angiogenic factors. A recent
study by Bowman and Bost [39] has shown that macrophage and dendritic cells are rich sources of the COX-2 protein in mesenteric lymph nodes of mice following infection with Salmonella. Use of the COX-2-specific inhibitor celecoxib to treat mice with salmonellosis resulted in prolonging the survival of lethally infected animals.

In the present study, we report that LPS and pro-inflammatory cytokines such as IL-1β and TNF-α induce expression of the COX-2 protein and mRNA from human monocytes as assessed by either Western or Northern blot analysis. The addition of either CC-5013 or CC-4047 inhibited the expression of COX-2 in a dose-dependent manner (Fig. 1A). However, the addition of these compounds at similar concentrations had no effect on the expression of the COX-1 protein, which suggests the specificity of the inhibitory activity of IMiDs on COX-2. In resting unstimulated PBMC, only COX-1 but not COX-2 was detected. Using the LPS-stimulated mouse cell line RAW 264.7, Fujita et al. have shown that thalidomide inhibited the expression of COX-2 from these cells.

Several studies have indicated that overexpression of COX-2 occurred in a variety of human malignancies, including colon, breast, lung, liver, skin, bladder, uterus, prostate, and ovarian cancers [40–42]. Furthermore, the COX-2 expression has been shown to promote angiogenesis, tumor invasion and metastasis [33,34]. Mao et al. isolated alveolar macrophages from heavily active smokers who were treated with the COX-2-selective inhibitor celecoxib and compared them with non-smokers for their COX-2 expressions. Their results indicated that treatment with a COX-2 inhibitor significantly reduced PGE2 (a product of COX-2) production from alveolar macrophages recovered from patients. This suggests that COX-2 inhibitors are able to modulate the microenvironment within the lungs and block the growth and metastasis of tumors. Using COX-2 inhibitors, numerous reports have shown that a decrease in COX-2 activity resulted in a decrease in tumor growth in several tumors [43,44]. Chen et al. [36] have shown that tumor invasiveness and liver metastasis of colon cancer cells correlated with COX-2 expression and were inhibited by a COX-2-selective inhibitor, Etodolac. Combination use of COX-2 inhibitor, celecoxib with chemotherapeutic agents such as CPT-11 and 5-fluorouracil have been shown to improve the cancer patient’s quality of life [45]. Recently, published results showed that a combination of CPT-11 with COX-2 inhibitors such as celecoxib or thalidomide enhanced the anti-tumor efficacy and reduced side effects of CPT-11 [46]. Combination therapy of thalidomide and CPT-11 in nine colorectal patients indicated that thalidomide was able to reduce the dose-limiting gastrointestinal toxic effects of CPT-11, especially diarrhea and nausea. This demonstrates the benefit and potential use of a combination of COX-2 inhibitors with other chemotherapeutic agents for cancer therapy.

Our data show that the decrease in COX-2 protein in response to IMiDs correlated well with a decrease in the expression COX-2 mRNA, as measured by Northern blot analysis (Fig. 2B). This decrease in response to IMiDs was detected after 4 h of drug treatment, suggesting that the drug is not affecting transcription of the COX-2 gene in stimulated monocytes. For this reason, we investigated the role of other proteins that might have been involved in the inhibitory process of CC-5013 on the expression of COX-2.

Regulation of gene expression in eukaryotes occurs at multiple levels [47]. Our data indicate that CC-5013 caused COX-2 mRNA degradation (Fig. 2C), which were consistent with the results for Fugita et al. [48].

In general, the IMiDs have an inhibitory effect on cytokines secreted by stimulated monocytes, but it has a stimulatory effect on cytokine production by stimulated T cells. The mechanism by which IMiDs inhibit the secretion of monocyte products such as TNF-α has been shown to be related to mRNA destabilization [13]. However, the effect of IMiDs on T cell cytokine production has been shown to be at the level of transcription, via increased activity of the transcription factor AP-1 [49]. In this regard, the effects of IMiDs on monocytes appear to be distinct from their effects on T cells. Anti-inflammatory cytokines such as IL-10 and IL-4 have been shown to down-regulate the expression of COX-2 from LPS-stimulated monocytes [50]. Also, Berg et al. [24] demonstrated the significant role of IL-10 in the regulation of COX-2 using IL-10-deficient mice. Our results indicate that CC-5013 enhanced the production of IL-10 in a dose-dependent manner compared with the DMSO control (Fig. 4B). Using Northern blot analysis, we also showed that IL-10 mRNA was detectable as early as 2 h and peaked at 8 h after stimulation (Fig. 4A). The addition of the anti-IL-10 antibody to treated PBMC increased the expression of COX-2 mRNA and protein (Figs. 5A and C). This demonstrates that CC-5013-mediated IL-10 production contributes to its inhibition of COX-2 expression.

A growing body of experimental evidence suggests that COX-2 plays an important role in the development of inflammation and the number of cancers. Furthermore, inhibition of COX-2 has been shown to reduce the growth of cancer cells in an in vitro and in vivo experimental setup. In conclusion, we have shown that thalidomide and IMiDs inhibit the expression of the COX-2 protein and mRNA from stimulated human PBMC. This opens the possibility that one of the mechanisms by which IMiDs have shown success in the treatment of oncological and inflammatory diseases is by inhibition of the COX-2 expression. Furthermore, we have shown that enhancement of IL-10 production by IMiDs plays a partial role in this COX-2 inhibitory process.
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References
