Interferon-α resistance can be reversed by inhibition of IFN-α-induced COX-2 expression potentially via STAT1 activation in A549 cells

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Abstract. The current study demonstrates that COX-2 expression is positively regulated by IFN-α, which is mediated by activation of STAT1 in A549 cells. The IFN-α-induced COX-2 expression and STAT1 activation were markedly inhibited by the addition of curcumin to the IFN-α-pretreated cells. While IFN-α or COX-2 inhibitors alone did not result in growth inhibition of A549 cells, the combination of IFN-α and celecoxib or curcumin resulted in a significant growth inhibition of A549 cells, which was associated with down-regulation of CDK2, 4, and 6 and up-regulation of p27.

We demonstrate that the expression of COX-2 was induced by IFN-α possibly via STAT1 activation in the A549 human non-small cell lung cancer cell line, which may partly account for its IFN-α resistance. The addition of curcumin or celecoxib to the IFN-α-pretreated A549 cells altered the IFN-α sensitivity of cell growth inhibition.

Introduction

Non-small-cell lung cancer (NSCLC) has, since the start of the new millennium, been the leading cause of cancer-related mortality in Korea as well as in Western countries (1). Despite advances in treatment, such as the development of new chemotherapeutic regimens, it has been suggested that a therapeutic plateau may have been reached (2). Various kinds of molecular mechanisms in the pathogenesis of lung cancer provide new opportunities for molecular-targeted therapies (3). One of the possible targets currently being evaluated in the treatment of lung cancer is cyclooxygenase-2 (COX-2), since the overexpression of COX-2 is commonly seen in human NSCLC (4-9). Studies indicate that overexpression of COX-2 is associated with apoptosis resistance, angiogenesis, decreased host immunity and enhanced invasion, metastasis, and thus poor prognosis (10,11). Therefore, considerable attention has focused on the mechanism and potential clinical implications of the COX-2 overexpression in cancer research over the past decade.

JAK (Janus tyrosine kinase)-STAT (signal transducers and activators of transcription) signaling, originally identified as the signaling pathway for interferons, mediates the immune responses of various cytokines as well as the action of various hormones and thus participates in inflammation (12). The interferon (IFN)-induced JAK-STAT pathway, a prototype signaling cascade for various cytokines, hormones, and growth factors, involves IFN-bound receptors activating JAK, which in turn activates cytoplasmic STAT via phosphorylation on a conserved COOH-terminal tyrosine residue (701). Active STAT1 dimers are then formed through reciprocal interactions between the tyrosine phosphorylated residue and the SH2 domain of the STAT1 monomer, triggering nuclear localization (13,14). Nuclear STAT1 dimers are known to bind specific sequences in the promoters of specific target genes, thus regulating the transcription of inflammation-associated genes (15). Among the four JAKs and seven STATs described to date, JAK1 and STAT1 are critical for both IFN-α/β and IFN-γ-driven responses (16). Recently, JAK-STAT signaling pathway has been recognized to play an essential role in the up-regulation of COX-2 protein during cardioprotection upon an ischemic assault (17).

Since the promoter of the COX-2 gene contains the interferon-γ activation site (GAS) consensus sequence, the site for the binding of STATs (18-20), we tested the hypothesis that COX-2 protein is overexpressed in lung cancer by activating the STAT1 pathway. Recently, we have shown that A549 cells,
a human non-small cell lung cancer cell line, were resistant to IFN-α, which was postulated to be caused by IFN-α-induced COX-2 activation (21). The current study demonstrates that COX-2 expression is positively regulated by IFN-α, which is potentially mediated by activation of STAT1 in A549 cells. The IFN-α-induced COX-2 expression and STAT1 activation were markedly inhibited by the addition of curcumin to the IFN-α-pretreated cells. While IFN-α or COX-2 inhibitors alone did not result in growth inhibition of A549 cells, combination of IFN-α and celecoxib or curcumin demonstrated an anti-proliferative effect.

Materials and methods

Tumor cell line and culture. Human non-small cell lung cancer cell line A549 was purchased from the American Type Culture Collection (Manassas, VA), cultured, and maintained in DMEM medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum, penicillin, and streptomycin. A549 cells were seeded on 60-mm dishes or 100-mm dishes at 3x10^5 cells per plate, or 8x10^5 cells per plate, respectively.

Cell viability assay. The in vitro growth inhibition effects of IFN-α, curcumin, and/or celecoxib on A549 cells were determined by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye absorbance of living cells (22). Briefly, cells (3x10^5 cells per well) were seeded in 96-well microtiter plates (Nunc, Roskilde, Denmark). After exposure to the drug for 72 h, 5 mg/ml MTT (Sigma) solution was added to the culture medium (10 μl per 100 μl of medium), and the plates were incubated for an additional 4 h at 37°C. MTT solution in medium was aspirated off. To achieve solubilization of the formazan crystal formed in viable cells, 100 μl of acid-isopropanol (0.04 N HCl in isopropanol) was added to each well before absorbance at 540 nM was measured.

Antibodies and reagents. Anti-STAT1, STAT3, STAT5, anti-CDK2, anti-CDK4, anti-CDK6, anti-COX-2, and anti-p27 antibodies were purchased from Santa Cruz Biotechnology [STAT1: stat1\p16 (c-111), Santa Cruz, CA]. Anti-phospho-STAT1 (Tyr701) antibody was purchased from Cell Signaling (Beverly, MA). Interferon-α2a was kindly provided by LG Life Sciences (Seoul, Korea). Curcumin and celecoxib were purchased from Sigma-Aldrich (St. Louis, MO).

Western blot analysis. Total cell extracts were obtained using ice-cold RIPA buffer (0.5% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris (pH 7.5), 0.1% SDS, 1 mM PMSF). After 20-min incubation on ice, the cell extracts were centrifuged for 20 min at 12,000 x g at 4°C and the supernatant was collected. Proteins were quantified with the BCA protein assay kit (Pierce, Rockford, IL, USA). For reverse transcription (RT)-PCR, 2 μg of RNA was treated with RNase-free DNase, and cDNA was obtained.
Preparation of nuclear protein extracts

Relative amounts of β-actin transcripts.

The cells were centrifuged (30 sec, 4˚C) to give a final concentration of 0.5%, and the cells were vortex mixed for 30 min at 4˚C. After centrifugation (5 min, 4˚C, 12,000 x g) the supernatant (= cytoplasmic extract) was frozen immediately and the nuclear pellets were resuspended in 130 µl buffer containing 20 mM Hepes (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF and vortex mixed for 30 min at 4˚C. After centrifugation (5 min, 4˚C, 12,000 x g) the supernatant (= nuclear extract) was frozen at -70˚C.

Electrophoretic mobility shift assay (EMSA). Specific STAT1 oligonucleotide form annealing the oligonucleotide sequence with the antisense strand, was purchased from Santa Cruz Inc. (Santa Cruz, CA). An oligonucleotide containing GAS binding sites in the human COX-2 promoter region (5'-TCT CTT TCC AAG AAA CAA G-3') was synthesized. The probe was purified and labeled with (γ-32P) ATP using T4 polynucleotide kinase. Labeled oligonucleotides (10,000 cpm), 10 µg of nuclear extracts, and binding buffer [10 mM Tris-HCl (pH 7.6), 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng of poly(dI-dC), and 1 mM dithiothreitol] were incubated for 30 min at room temperature in a final volume of 20 µl. The reaction mixture was analyzed by electrophoresis on a 5% polyacrylamide gel in 0.5× tris-borate/EDTA buffer. Specific binding was controlled by competition with a 50-fold excess of cold oligonucleotides. For supershift/inhibition assay, 1-2 µg of specific supershifting antibodies against STAT1 were incubated with the nuclear extract on ice for 1 h before the addition of labeled oligonucleotide to the binding reaction.

Analysis of DNA fragmentation. The A549 cells (3x10^5 cells/well) were exposed to 1000 U/ml IFN-α with or without celecoxib 50 M or 25 M curcumin. At 72 h, cells treated with indicated drug were washed twice with PBS (4˚C) and subsequently DNA isolation was performed using the ApopLadder kit (TaKaRa, Korea). Gel electrophoresis was performed in 2.0% agarose (Sigma-Aldrich Corp.) containing ethidium bromide, using 100-bp DNA ladder as a marker (TaKaRa).

Results

Effect of IFN-α on phosphorylation and DNA binding activity of STAT1. We analyzed the IFN-α-induced phosphorylation of STAT1, which is a prerequisite for STAT dimerization and, thus, activation of the protein. We examined the IFN-α-induced phosphorylation of STAT1 protein by Western blot analysis, and found that the maximum phosphorylation of STAT1 protein was observed at 15 min, with a rapid return to the basal levels of phosphorylation at 120 min (Fig. 1A). The nuclear extracts were analyzed by EMSA using a 32P-labeled STAT1 oligonucleotide probe. As illustrated in Fig. 1B, IFN-α induced an increase in the STAT-DNA complex with maximum DNA binding activity of STAT1 at 30 min. Therefore, the increased phosphorylation of nuclear STAT1 was associated with the corresponding increase in STAT-DNA binding activity. In order to determine the effect of IFN-α on other STAT proteins, we performed Western blotting and RT-PCR using antibodies against STAT3, STAT5 and STAT2-, STAT3-, STAT4-, STAT5-specific primers, respectively (Table I). The mRNA levels of STAT2, STAT3, STAT4, and STAT5 and the protein expression levels of STAT3 and STAT5 were not increased after IFN-α.
treatment (Fig. 2). Therefore, there was a notable increase specifically in STAT1 mRNA and protein expression levels after IFN-α treatment.

**Induction of COX-2 expression by IFN-α.** To determine whether activation of STAT1 is involved in the regulation of COX-2 expression, A549 cells were incubated with 1000 U/ml IFN-α for an indicated time and RT-PCR using COX-2 specific primers (Table I) was performed (Fig. 3A). Fig. 3A shows that expression of both STAT1 and COX-2 mRNA was up-regulated in response to 1000 U/ml IFN-α exposure in a time-dependent fashion. To determine if the observed increase in COX-2 mRNA transcription correlates with COX-2 and STAT1 protein expression, we performed Western analysis after treatment with IFN-α for an indicated time (Fig. 3B). After 1000 U/ml IFN-α treatment for 12 h, the COX-2 protein expression was augmented by approximately

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**Figure 2.** Effect of IFN-α on other STAT proteins. The mRNA levels of STAT2, STAT3, STAT4, and STAT5 and the protein expression levels of STAT3 and STAT5 were not increased after 1000 U/ml IFN-α treatment for 12 h.

**Figure 3.** IFN-α-induced STAT1 activation and COX-2 expression. (A) The COX-2 and STAT1 protein expression was measured by Western immunoblotting after treating A549 cells with 1000 U/ml IFN-α for different amounts of time. (B) The mRNA expression levels of COX-2 and STAT1 were analyzed using RT-PCR.

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**Figure 4.** Binding of STAT1 to the GAS nucleotide sequences of COX-2. (A) A549 cells were treated with different concentrations of IFN-α for 30 min, and the nuclear extracts were prepared and assayed for COX-2-GAS by EMSA. (B) Nuclear extracts were obtained from A549 stimulated without (lane 2) or with 1000 U/ml IFN-α for 30 min (lanes 3-5), and then incubated with the 32P-labeled COX2-GAS probe. Lane 4, the probe was incubated with anti-STAT1 antibody in the assay mixtures and showed supershifting of the band (arrow). (C) Supershifting of the IFN-α complex was not induced by the addition of anti-STAT3 antibody.
three-fold (Fig. 3B). We also have previously reported that IFN-α-induced COX-2 protein expression in a concentration-dependent manner in A549 cells (21). Collectively, these data suggest that IFN-α possibly induces COX-2 and STAT protein expression in A549 cells by enhancing transcriptions of the COX-2 and STAT1 genes.

**Binding of STAT1 to the GAS nucleotide sequences of COX-2.** Since the promoter of the COX-2 gene contains the GAS consensus sequence, which is known to bind STATs (19,22,23), we hypothesized that activated STAT1 binds to the GAS motif sequence of COX-2 following IFN-α treatment. After treating cells with 100, 500, and 1000 U/ml IFN-α for 30 min, nuclear extracts were analyzed by EMSA. IFN-α treatment resulted in a concentration-dependent increase in the COX2-GAS binding complex (indicated by arrow, Fig. 4A). In order to assess whether STAT1 directly binds to the GAS probe within the COX-2 promoter, the anti-STAT1 antibody was added to the binding mixture (lane 4, Fig. 4B). The identity of the IFN-α-induced complex was confirmed by shifting with anti-STAT1 antibody (Fig. 5B), but not with normal rabbit serum (data not shown). These results suggest that STAT1 potentially mediates IFN-α-induced COX-2 upregulation by binding to the GAS element. The addition of anti-STAT3 antibody did not result in supershifting of the complex, which confirms the specificity of the binding activity (Fig. 5C).

**Curcumin inhibits IFN-α-induced COX-2 expression via STAT1 inactivation.** We investigated whether curcumin (diferuloylmethane), a phenolic compound from the plant, Curcuma longa (Linn), with anti-inflammatory and anti-tumor activities, inhibits IFN-α-induced COX-2 expression via STAT1 inactivation. Curcumin inhibited IFN-α-induced NF-κB and COX-2 expression in A549 cells (21). Recent studies also showed that the anti-inflammatory effects of curcumin were related to the suppression of JAK-STAT activation in murine microglial cells (23). To examine the inhibitory effect of curcumin on phosphorylation of STAT1 in IFN-α-stimulated A549 cells, A549 cells were stimulated with 1000 U/ml IFN-α for different times with or without 25 μM curcumin treatment. Curcumin abrogated the phosphorylation of STAT1 in stimulated A549 cells with IFN-α in a time-dependent manner (Fig. 5A, right panel). Next, we assessed the effect of curcumin on COX-2 and STAT1 protein expression after IFN-α stimulation. As shown in Fig. 5B, STAT1 protein expression was augmented after treating cells with 1000 U/ml IFN-α for 60 min (lanes 1 and 2, Fig. 5B). After pretreatment with increasing concentrations of curcumin, STAT1 and COX-2 protein expression was decreased in a concentration-dependent manner (lanes 3-5). We further examined whether phosphorylated STAT1 binds to GAS motifs and whether curcumin suppresses the IFN-α-induced STAT1 binding activity. As illustrated in Fig. 5C, IFN-α-induced STAT1-DNA binding activity was significantly abrogated by curcumin in a concentration-dependent manner, which further supported the IFN-α-induced COX-2 expression via STAT1 activation in A549 cells.

**Celecoxib and curcumin plus IFN-α inhibited cell growth of A549 cells.** We investigated whether celecoxib or curcumin, which suppresses COX-2 activation, could rescue A549 cells from their resistance to IFN-α treatment by inhibiting IFN-α-induced COX-2 expression. Whereas exposure of cells to IFN-α or celecoxib alone for 72 h did not inhibit cell proliferation (cell viability >80%), cell growth was prominently inhibited when both celecoxib (Fig. 6A) or curcumin (Fig. 6B) and IFN-α were added in combination (cell viability <40%). Collectively, while IFN-α or COX-2
inhibitors alone did not result in growth inhibition of A549 cells, combination of IFN-α and celecoxib or curcumin demonstrated an anti-proliferative effect.

Anti-proliferative effect of IFN-α plus celecoxib or curcumin was not apoptotic. To determine whether the growth inhibition effect of IFN-α plus COX-2 inhibitors involved apoptosis, DNA fragmentation analysis and nuclear DAPI staining were used. Agarose (2%) gel electrophoresis of extracted genomic DNA from both control (HL-60 cells treated with 100 M etoposide) and IFN-α/COX-2 inhibitor-treated cells was performed (Fig. 7A and B). No typical 180-bp oligonucleosome ‘DNA ladder’ that would be indicative of intranucleosomal cleavage was detected in any samples, suggesting that the growth inhibition did not occur through apoptosis. Nuclear DAPI staining was carried out to examine any morphologic changes in the nuclei of A549 cells treated with IFN-α and celecoxib or curcumin. There were no typical nuclear changes associated with apoptosis, such as fragmented apoptotic bodies (data not shown). To investigate the effect of combination of IFN-α and celecoxib or curcumin on cell cycle regulatory proteins, we examined the expression levels of CDK2, CDK4, and CDK6, which are associated with the regulation of G1 progression and G1/S transition. The treatment of A549 cells with IFN-α alone did not alter the expression levels of CDK2, 4 and 6 (Fig. 7C, lanes 1-2). However, the CDK2, 4, and 6 expression levels were decreased when 10 M, 25 M, and 50 M celecoxib was added to A549 cells in combination with 1000 U/ml IFN-α (lanes 2, 4, 6, 8). When 10 M and 25 M curcumin were added to IFN-α-treated A549 cells, the expression of CDK2, CDK4, and CDK6 was also significantly down-regulated (Fig. 7C).

We further examined the effect of celecoxib and curcumin on the protein levels of CDK inhibitors, p27, in A549 cells...
which were exposed to IFN-α. As illustrated in Fig. 7C, the expression levels of p27 were enhanced in cells exposed to a combination of celecoxib or curcumin and IFN-α. Collectively, these data suggest that the combination of celecoxib or curcumin and IFN-α is likely to result in cell cycle arrest at G1/S phase.

**Discussion**

Our recent study demonstrated that the exposure of A549 lung cancer cells to IFN-α did not result in growth inhibition, which may partially be explained by IFN-α-dependent activation of NF-κB and COX-2 (21). The activation of NF-κB and COX-2 by IFN-α was markedly abrogated by the addition of curcumin (21). In this study, we further showed that STAT1 was also activated by IFN-α treatment, which was nearly completely inhibited by the addition of curcumin in A549 cells. The combination of IFN-α and COX-2 inhibitors, curcumin or celecoxib, resulted in growth inhibition of A549 cells while either IFN-α or COX-2 inhibitors alone did not.

Although several mechanisms have been postulated for COX-2 overexpression in tumor cells, none of the earlier
studies have demonstrated that IFN-α could induce COX-2 expression via STAT1 activation. We demonstrated that IFN-α-treated A549 cells resulted in phosphorylation and activation of STAT1 protein (Fig. 3). The involvement of STAT protein in COX-2 expression has been recently documented in ischemic pre-conditioning of the myocardial cells (17), but has not been reported in tumor cells. Active STAT1/STAT2 heterodimers translocate into the nucleus and subsequently enter the nucleus and bind IFN-stimulated response elements. Although the role of curcumin on STAT1 activation has not been fully elucidated in tumor cells, curcumin attenuated inflammatory response by inhibiting JAK-STAT1 signaling in activated microglial cells (23). We observed that curcumin reduced the phosphorylation of STAT1 as well as IFN-α-induced STAT1-DNA binding activity. Our study coincides with a previous report, which demonstrated that curcumin suppressed the IFN-α-induced STAT1 phosphorylation in myeloma cells (18).

Several pathways have been proposed for escape mechanisms to the antitumor effects of IFN-α (25). IFN-α induces different survival pathways which may protect tumor cells from apoptosis. Recently, Caraglia and colleagues have reported that IFN-α increases epidermal growth factor receptor (EGFR) expression and activates its downstream targets including the Ras/ERK (extracellular signal-regulated kinase) pathway, thereby protecting cells from apoptosis (26). Other proposed escape mechanisms include activation of the STAT3/PI3-K (phosphoinositide kinase-3)-dependent pathway (27), stimulation of Shp2 (SH-2 containing protein-tyrosine phosphatases 2) (28), and induction of the ubiquitin cross-reactive protein inactivating NF-xB (29). The mechanism of IFN-α resistance in some solid tumors and inconsistent treatment outcome of IFN-α may be explained by COX-2 overexpression. COX-2 overexpression may result in tumor proliferation, invasion, angiogenesis, and resistance to apoptosis (10,11). COX-2 inhibitors have shown to inhibit tumor growth in animal models and have demonstrated responses when combined with cytotoxic therapy in phase II clinical trials (11). There was a significant increase in COX-2 protein expression in IFN-α-resistant bladder cancer tissue (30). The increased expression of COX-2 with IFN-α treatment in cancer cell lines may propose the potential application of the combination therapy of COX-2 inhibitors and IFN-α, although in vivo studies should proceed.

Combination of IFN-α and curcumin resulted in down-regulation of CDKs 2, 4, 6 and up-regulation of p27, suggesting that cells are arrested at G1/S phase. While most COX-2 inhibitors primarily induce apoptosis, others may predominantly induce growth arrest and may induce anti-tumor effects via different mechanistic pathways in different cell types (11,31). Hung et al found that p27kip1 was upregulated in response to the COX-2 inhibitor, NS398, and thus inhibited the cell cycle in human lung cancer cells (32). Recent cDNA microarray analysis showed that curcumin modulated cell cycle related gene expression in human umbilical vein endothelial (ECV304) cells in vitro (33). Moreover, curcumin inhibited constitutive NF-xB activation and induced G1/S arrest in mantle cell lymphoma cells (34). Celecoxib, a COX-2 selective inhibitor, also inhibited cell growth and altered cell cycle-regulatory proteins in IFN-α-treated A549 cells in the present study. Therefore, the addition of COX-2 inhibitors to IFN-α treatment may target the escape mechanism and thus may widen the therapeutic application of IFN-α.

We demonstrate that the expression of COX-2 was potentially induced by IFN-α via STAT1 activation in the A549 human non-small cell lung cancer cell line, which may partly account for its IFN-α resistance. The addition of curcumin or celecoxib to the IFN-α pretreated A549 cells altered the IFN-α sensitivity of cell growth inhibition. We further observed that the addition of curcumin or celecoxib not only suppressed COX-2 expression but also modulated cell cycle-regulatory proteins, such as CDK2, CDK4, CDK6 and p21, which may play an important role in overcoming the IFN-α resistance of A549 cells. The association between the overexpression of COX-2 and IFN-α treatment needs further investigation, especially in tumor types in which interferon therapy is widely used.

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References


