

Prostaglandin E₂-Dependent Activation of Src-Stat3 Signal Pathway in Human Lung Adenocarcinoma Cells

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Abstract: Accumulating evidence suggests that overproduction of prostaglandin (PG) E₂ attributable to induction of cyclooxygenase-2 plays an important role in the development of lung adenocarcinoma. Recently, we have reported that a PGE₂ receptor, EP3 is involved in appearance of malignant phenotype of a lung adenocarcinoma cell (A549 cell). In line with our previous study, here we investigated if Src signaling could be involved in PGE₂-stimulated growth of A549 cells via EP3. PGE₂-dependent cell growth in A549 cell positively related to the activation of Src. A specific antagonist against EP3 abrogated the cell growth and Src activation in the cells stimulated with PGE₂. Also, the inhibition of Src activity suppressed its downstream signaling related to cell growth as well as the cell growth in the cells treated with PGE₂. These results indicate that PGE₂-dependent activation of Src signaling via EP3 plays an important role in growth of A549 cells.

Keywords: PGE₂; Src; STAT3; EP3; lung adenocarcinoma

INTRODUCTION

Lung cancer, particularly non-small cell lung cancer (NSCLC), is one of the most common cancers and the leading cause of cancer death in Western countries as well as in Japan [1, 2]. Of NSCLC, adenocarcinoma type is the majority of the histology [3]. Recently, it has been notable that cyclooxygenase-2 (COX-2) is a useful diagnostic marker and target for prevention and therapy of lung adenocarcinoma [4, 5]. In fact, the inhibition of COX-2 activity contributes to the blockage of growth in lung adenocarcinoma cells including A549 cell [6]. The induction of COX-2 is associated with high generation of prostaglandin E₂ (PGE₂) in the lung adenocarcinoma cells, and the high generation stimulates the growth of the cells [6]. Thus, to determine the PGE₂-regulated growth signaling may a key clue to clarify the detailed mechanism of PGE₂-dependent appearance of malignant phenotypes in lung adenocarcinoma cells.

PGE₂ induces its effects, mainly through G protein-coupled PGE receptor designated EP1, EP2, EP3, and EP4 [7]. Differential expression of these EP receptors mediates the diverse and often antagonistic effects of PGE₂ and its analogues on a variety of cell types [8, 9]. Recently, we have reported that the expression of EP3 is detected in A549 cell but not alveolar type II cell (a main progenitor cell of lung adenocarcinoma) and that the expression contributes to cell growth in the lung adenocarcinoma cell [10]. Furthermore, other recent report has indicated that PGE₂-EP3 signaling appears critical for tumor-associated angiogenesis, tumor progression and tumor growth in a mouse tumor implantation model [11]. Overall, it seems to be possible that EP3 plays a pivotal role in the development of the tumors. In a recent report, it has been demonstrated that the proto-oncogene product, pp60c-src (Src), which is a member of non-receptor tyrosine kinase, is activated in lung adenocarcinoma and that the activation plays a criti-

cal role in the progression of the adenocarcinoma [12]. Also, the activation of signal transducers and activators of transcription (Stat) 3 by Src regulates survival in lung adenocarcinoma cells [13]. These reports mean that Src-Stat3 signaling closely relates to the appearance of malignant phenotypes in lung adenocarcinoma cells. On the other hand, recent studies have suggested that heteromeric guanine nucleotide-binding regulatory protein (G protein), especially Gi coupled to receptors, causes proliferation and transformation in cells through Src-Stat3 signal pathway [14, 15]. Since EP3 is coupled to Gi [16], it is likely that PGE2-dependent activation of EP3 is associated with the activation of Src-Stat3 signaling in A549 cells. In the context, this study was undertaken to estimate if PGE2 could activate Src signaling through EP3 and stimulate the growth based on the activation in A549 cells.

EXPERIMENTAL

Cell culture and treatment. A human lung adenocarcinoma cell line, A549 cell was provided by Riken Cell Bank (Saitama, Japan). This cell was routinely grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin. For experiments, exponentially growing cells were used. Cells (4×10^5 cells per plate) were plated on culture plates and cultured for 24 h to permit adhere. After attachment, in order to arrest cell growth, 48 h starvation with low serum condition (0.2% FCS) was carried out. After the starvation, PGE2 (Calbiochem, San Diego, CA) was added to culture medium. Pertussis toxin (PTX: Calbiochem) and PP1 (Calbiochem) treatment were performed for 4 h before the stimulation of PGE2. Control was treated with vehicle only. Cell proliferation assay was examined by WST-1 assay kit (Quick Cell Proliferation Assay Kit, MBL, Nagoya, Japan), according to manufacturer's instruction.

Assay of Src and Stat3 activation. The activation of Src and Stat3 was estimated by immunoblot analysis using a specific antibody against each phosphorylated form. In brief, the cells were lysed in 1 ml of ice-cold lysis buffer (50mM HEPES, pH 7.5, 150mM NaCl, 10% glycerol, 1mM EDTA, 1% Triton X-100, 10mM β -glycerol phosphate, 0.1mM sodium vanadate, 1mM NaF, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 50 μ g/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 1mM dithiothreitol fluoride hydrochloride, 1mM dithiothreitol). The

lysates were separated on 10% SDS-PAGE, transferred to nitrocellulose membranes and subjected to immunoblotting with anti-phosphorylated Src and anti-Src (UBI, Lake Placid, NY, USA), or anti-phosphorylated Stat3 and anti-Stat (Santa Cruz Biotechnology, Santa Cruz, CA). Detection was accomplished using the ECL system (Amersham, Piscataway, NJ, USA) and a cooled CCD camera-linked Cool Saver System (Atto, Tokyo, Japan). A two-dimensional densitometric evaluation of each band was performed using ATTO Image Analysis Software (ATTO). Molecular sizing was done using Rainbow Molecular Weight Marker (Amersham, Piscataway, NJ). Protein concentration was determined using DC Protein Assay (Bio-Rad, Hercules, CA).

siRNA design, preparation and transfection. The design of short interfering RNA (siRNA) was carried out by an on line design system for siRNA (Qiagen, Germantown, MD). siRNAs were synthesized in High-Performance Purity Grade by Qiagen. The sense and anti-sense strands of Stat3 siRNA were: sense: 5'-AACAUUCUGCCUAGAUCG-GCUAdTdT-3'; anti-sense: 5'-UAGCCGAUCUAG-GCAGAUGUdTdT-3'. The sense and anti-sense strands of nonspecific control siRNA were: sense: 5'-UUCUCCGAACGUGUCACGUdTdT-3'; anti-sense: 5'-ACGUGACACGUUCGGAGAAAdTdT-3'. A549 cells were seeded into 6-well plates one day prior to transfection in DMEM medium supplemented with 10% FCS. At the time of transfection with siRNA (4 μ g/well), the cells were 70% confluent. RNAiFect Transfection Reagent (Invitrogen) was used as the transfection agent, the cells were then incubated for 48 h in normal growth medium including siRNA, and subsequently each protein level was determined by immunoblot analysis.

Other assay. The level of each protein related to cell growth (cyclinD) and apoptosis (Bcl-2, Bcl-xL and Bax) was estimated by immunoblot analysis using anti-cyclinD, Bcl-2, Bcl-xL and Bax antibodies (BD Bioscience, San Jose, CA) as mentioned above.

RESULTS AND DISCUSSION

Initially, we estimated the relation between cell growth stimulation and Src activation in A549 cells treated with PGE2. In this assessment, we used a culture condition with low serum level (serum concentration, 0.2%) in order to maintain attachment of the cells for culture dishes. The activation of Src was caused by PGE2 treatment. In similar with this activation, cell growth of A549 cells was

also stimulated by the treatment. These results suggest that the PGE2-dependent cell growth of A549 cells is associated with the activation of Src. PGE2 receptors are members of G protein-linked receptors [17], and the expression patterns of the receptors in each cell govern diversity of cell responses in reply to PGE2 stimulation [8, 9]. We have previously reported that A549 cell has the expression of EP3 [10], and the PGE2 receptor has been generally known to be coupled to Gi protein, which induces the activation of Src [18]. Thus, it is likely that the expression of EP3 causes the Src activation in A549 cells treated with PGE2.

Of the known PGE2 receptors, EP3 has been shown to be coupled to PTX-sensitive G protein (Gi) [7], so we used PTX as an antagonist against EP3 to confirm a central role of the receptor in Src activation. PGE2-dependent activation of Src was abrogated by PTX treatment, but only PTX treatment did not give any effect on the activation. In link with this event, PGE2-stimulated cell growth was reduced by the PTX treatment with a statistical significance. Also, only the PTX treatment had no influence on cell growth. These results clearly demonstrate that EP3 contributes to the activation of Src and its related cell growth in A549 cells treated with PGE2.

Finally, we tried to determine the relation between Src signaling and cell growth in A549 cells stimulated with PGE2, and estimate the Src-governed signaling related to cell growth and survival. A Src specific inhibitor (PP1) inhibited PGE2-dependent cell growth in A549 cells, but only PP1 treatment has less inhibitory effect on the cell growth. Since recent studies show that signal of Gi-mediated transformation in cells is regulated by Src and Stat3 [14, 18], we estimated whether Stat3 located in the downstream of Src signaling in A549 cells. As the result, the inhibition of PGE2-dependent Src activation by PP1 induced the inactivation of Stat3, indicating that Src is required for the activation of Stat3 in A549 cells treated with PGE2. In order to further confirm the contribution of Src-Stat3 signaling to PGE2-stimulated cell growth in A549 cells, we tried to determine cell growth and survival-related molecules down-regulated by silencing of Stat3 gene. Short interfering RNA (siRNA) against Stat3 reduced the level of cyclin D and Bcl-xL, but the treatment had no effect on the levels of Bcl-2 and Bax in A549 cells. These data suggest that cyclin D and Bcl-xL are important downstream targets of the Src-Stat3 signal pathway in A549 cells.

CONCLUSION

The idea for targeting EP3-Src signaling leads to the development of new agents for lung adenocarcinoma prevention and therapy.

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