Cyclooxygenase 2-Dependent Expression of Survivin Is Critical for Apoptosis Resistance in Non-Small Cell Lung Cancer

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Abstract

Elevated tumor cyclooxygenase 2 (COX-2) expression is associated with increased angiogenesis, tumor invasion, and promotion of tumor cell resistance to apoptosis. In our previous studies using non-small cell lung cancer (NSCLC) cell lines constitutively expressing COX-2 cDNA in sense and antisense orientations, we demonstrated that constitutive overexpression of COX-2 leads to stabilization of the inhibitor of apoptosis protein survivin resulting in the elevated apoptosis resistance of COX-2–overexpressing cells. Genetic or pharmacologic suppression of COX-2 activity increased proteasomal degradation of survivin and cellular response to apoptosis induction. Our data show that expression of survivin in non-small cell lung cancer cells can be significantly down-regulated by RNA interference. Whereas COX-2–overexpressing NSCLC cells have significantly higher apoptosis resistance than the parental cells, inhibition of survivin expression by small interfering RNA decreases apoptosis resistance to the level of the parental non-small cell lung cancer. We conclude that COX-2-dependent expression of survivin is critical for apoptosis resistance in non-small cell lung cancer.

Introduction

Cyclooxygenases are the rate-limiting enzymes in prostaglandin synthesis, which convert arachidonic acid into prostaglandin (PG) H2, a substrate for specific PG synthases (1). Two isoforms of cyclooxygenase (COX) have been isolated and characterized: the ubiquitously expressed the COX-1 and inducible COX-2 (2). Studies of human cancers have revealed frequent overexpression of COX-2 in a variety of different malignancies. High-level constitutive COX-2 expression has been detected in colorectal (3), head and neck (4), lung (3, 5, 6), breast (3), and other cancers. Overexpression of COX-2 and elevated production of the COX-2 metabolite PGE2 can stimulate epithelial cell growth and invasion and promote cellular survival (7).

Recent studies show that COX-2 promotes cell survival by modulation of antiapoptotic protein expression (8). Survivin is a member of the inhibitor of apoptosis protein (IAP) family and is able to bind caspases thus preventing their activation (9). Suppression of survivin expression leads to increased caspase-3 activation and apoptosis as well as mitosis deregulation and sensitization to anticancer drugs (10).

Our previous results indicate that COX-2 overexpression leads to stabilization of survivin in non-small cell lung cancer that alters the balance between pro- and antiapoptotic proteins and facilitates the prosurvival phenotype, resulting in elevated resistance to apoptosis (11).

In the present study, we investigated the effect of survivin inhibition by RNA interference in COX-2–overexpressing non-small cell lung cancer cell lines. These cell lines were used to model the COX-2–rich tumor microenvironment. We found that inhibition of survivin expression overcame the prosurvival phenotype of COX-2–overexpressing non-small cell lung cancer (NSCLC) cells. Inhibition of survivin in COX-2 sense tumor cells reduced the apoptosis threshold to the level of the parental cell lines. These findings indicate the importance of survivin in mediating COX-2–dependent apoptosis resistance in NSCLC.

Materials and Methods

Stable Transduction of Non-Small Cell Lung Cancer Cells. NSCLC cells A549 (human lung adenocarcinoma) and H157 (human squamous cell carcinoma) were obtained from American Type Culture Collection (Rockville, MD) and the National Cancer Institute (Bethesda, MD), respectively. A 2.0-kb cDNA fragment of human COX-2 (generously provided by Dr. Harvey Herschman, University of California at Los Angeles) was cloned in sense and antisense orientations in the retroviral vector pLNCX (Clontech, Palo Alto, CA) as described previously (11). For each cell line, an approximately 10-fold higher level of COX-2 expression and PGE2 production was noted in sense compared with parental or vector controls (7).

Inhibition of Survivin Expression by Small Interfering RNA (siRNA). Stable transduction of non-small cell lung cancer cells was performed with retroviral vector pLNCX (Clontech, Palo Alto, CA) at different RNA/transfection reagent ratios. At all conditions, we observed a significant suppression of survivin expression without any signs of cytotoxicity or notable decrease of actin levels as assessed by Western blotting (data not shown). For additional experiments transfection was performed in serum-free RPMI using 1.6 μg of survivin or control siRNA, 3.2 μg of Enhancer R, and 12 μL of TransMessenger reagent for 3 hours followed by the replacement of transfection medium with fresh RPMI supplemented with 10% fetal bovine serum. Cells were transfected with validated survivin or negative control Silencer siRNA (Ambion, Inc., Austin, TX) using TransMessenger transfection reagent (Qiagen, Valencia, CA) at different RNA/transfection reagent ratios. At all conditions, we observed a significant suppression of survivin expression without any signs of cytotoxicity or notable decrease of actin levels as assessed by Western blotting (data not shown). For additional experiments transfection was performed in serum-free RPMI using 1.6 μg of survivin or control siRNA, 3.2 μL of Enhancer R, and 12 μL of TransMessenger reagent for 3 hours followed by the replacement of transfection medium with fresh RPMI supplemented with 10% fetal bovine serum and incubation for an additional 24 hours. After 24 hours of incubation, apoptosis was induced, and the efficiency of survivin suppression was assessed by Western blotting or enzyme-linked immunosorbent assay (ELISA).

Apoptosis Induction and Detection Techniques. Apoptosis was induced by addition of 17 μmol/L Camptothecin (Sigma, St. Louis, MO) for 18 hours. After 18 hours, cells were stained with 0.1 ng/mL of apoptotic stain YO-PRO-1 (Molecular Probes, Eugene, OR) for 20 minutes and visualized by the fluorescent microscope (Leica Microsystems, Wetzlar, Germany). Photographs were taken from several randomly selected fields from each sample. Images were analyzed using the Scion Image software.

Western Blotting. Western blotting was performed as described previously (11). In brief, cells were washed with PBS and lysed with modified radioimmunoprecipitation assay buffer. Proteins were separated in SDS-PAGE and transferred to the nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). Membranes were blocked and incubated with antisurvivin anti-
bodies (Novus Biologicals, Littleton, CO) diluted 1:1000 in blocking solution followed by treatment with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:10,000 in blocking solution and developed using a SuperSignal West Pico kit (Pierce, Rockford, IL).

Survivin ELISA was performed using the DuoSet Human Survivin ELISA kit (R&D Systems, Minneapolis, MN) in the conditions recommended by the manufacturer. Protein concentration was determined in the cell lysates, and survivin concentration was calculated per milligram of the total protein.

**Statistical Analysis.** Probability values were calculated using two-tailed nonpaired Student’s *t* test.

**Results and Discussion**

Survivin Expression Is Effectively Suppressed by Ribonucleic Acid Interference in Non-Small Cell Lung Cancer Cell Lines. RNA interference is a powerful tool for inhibiting the expression of specific genes (12). Using Western blotting (Fig. 1A) and ELISA (Fig. 1B), we found that survivin expression was strongly suppressed by specific siRNA and not affected by the control siRNA, whereas the actin levels were unaffected by either siRNA. We did not observe any signs of transfection reagent-dependent cytotoxicity after the treatment and upon the subsequent incubation. Our data demonstrate high efficiency of the RNA interference technique in suppression of survivin expression in NSCLC cells.

**Inhibition of Survivin Expression by siRNA Sensitizes the Cyclooxygenase 2–Overexpressing Non-Small Cell Lung Cancer Cells to Apoptotic Stimuli.** The overexpression of survivin in tumors has been linked to poor prognosis and diminished patient survival in prostate (13), lung (14), and other cancers. Previous studies have shown that COX-2 and its metabolite PGE2 promote cancer cell survival (8). We have found previously that COX-2 overexpression leads to survivin stabilization via decreased proteasomal degradation that renders the NSCLC cells more resistant to apoptotic stimuli. In contrast, pharmacologic inhibition of COX-2 by specific inhibitors significantly reduces survivin levels and thus enhances susceptibility of NSCLC cells to radiation and chemotherapy agents (11). Consistent with the pharmacologic inhibition, genetic inhibition of COX-2 expression led to enhanced apoptosis sensitivity, whereas overexpression increased the apoptosis threshold.

To determine the importance of survivin in mediating COX-2-

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**Fig. 1.** Inhibition of survivin expression in NSCLC cells by RNA interference. A549 and H157 non-small cell lung cancer cells were transfected with control or survivin siRNA as described in Materials and Methods. A. Top section. Survivin expression was assessed after 24 hours by Western blotting. Bottom section. The blot was washed and reprobed with antiantibodies to ensure equal loading. B, survivin levels (picogram per microgram of total protein) by ELISA in parental (P) and COX-2 sense (S) A549 and H157 cells transfected with control or survivin siRNA.

**Fig. 2.** Inhibition of survivin expression significantly reduces apoptosis resistance of A549 NSCLC cells. Parental (P) and COX-2 sense (S) A549 non-small cell lung cancer cells were transfected with control or survivin siRNA and incubated for 24 hours. After 24 hours, apoptosis was induced by addition of Camptothecin for 18 hours. Cells were stained with the YO-PRO nuclear dye, which brightly stains apoptotic cells as indicated in Materials and Methods. A. Randomly selected fields were photographed under the fluorescent microscope. B. The fluorescence intensity was measured using Scion Image software.
expression inhibition by siRNA on apoptosis resistance of COX-2
dependent apoptosis resistance, we tested the effect of survivin ex-
overexpressing NSCLC cell survival. By specific inhibition
of survivin, we were able to overcome the antiapoptotic effect
of COX-2 overexpression. This finding is particularly important given
the fact that survivin levels are modulated by COX-2 expression. As
a COX-2–dependent factor of apoptosis resistance, survivin may be
an important target for antitumor therapy. Additional studies are
required to further define the importance of COX-2 and survivin in
lung cancer. In particular, additional studies will be required to
determine whether survivin can serve as a COX-2-dependent biomar-
ker in lung cancer clinical trials that utilize COX-2 inhibition.

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