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NS398, a Selective Cyclooxygenase-2 Inhibitor, Induces Apoptosis and Down-Regulates bcl-2 Expression in LNCaP Cells

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Abstract

Cyclooxygenase (COX)-2, an inducible enzyme that catalyzes the formation of prostaglandins and other eicosanoids from arachidonic acid, is constitutively expressed in LNCaP human prostate cancer cell line. To evaluate the potential role of COX-2 in prostate cancer, LNCaP cells were treated with NS398, a selective COX-2 inhibitor, and the effects on cell viability and apoptosis were determined. NS398 treatment induced apoptosis in LNCaP cells in a time- and dose-dependent fashion. Treatment with 100 \( \mu \)M NS398 caused a down-regulation in bcl-2 protein expression, followed by chromatin condensation, chromosomal DNA fragmentation, and changes in nuclear morphology detected by 4,6-diamidino-2-phenylindole staining, DNA fragmentation assay, and terminal deoxynucleotidyl transferase-mediated UTP-biotin nick end-labeling assay. In contrast, NS398 treatment had no effect on either cell viability or nuclear function and morphology in human fetal prostate fibroblasts. These results demonstrate that NS398 induces apoptosis in LNCaP cells but not in human fetal prostate fibroblasts, and that this induction is associated with a decreased level of bcl-2 protein.

Introduction

Prostate cancer is the most common malignancy of aging men and the second leading cause of male cancer deaths in the United States (1). Although most patients respond initially to androgen ablative therapies, the prostatic cells eventually and inevitably lose their sensitivity to androgen. Presently available treatments for advanced, hormone-resistant prostate cancer are only marginally effective, and newer agents are needed to selectively kill the cancer cells.

Apoptosis, or programmed cell death, is a highly regulated process that involves the activation of a cascade of molecular events leading to cell death that is characterized by plasma membrane blebbing, shrinkage, chromatin condensation, chromosomal DNA fragmentation, and formation of membrane-bound apoptotic bodies that are eventually phagocytized by neighboring cells (2). In the normal prostate, there is a balance between cell proliferation and cell death. It is well established that androgens act upon prostate epithelial cells by both stimulating their proliferation and inhibiting apoptosis (3). In prostate cancer, even in advanced, hormone-refractory disease, the proliferative rate remains relatively low, but there is less apoptosis (4). The identification, therefore, of agents that specifically induce apoptosis in prostate cancer cells may be a key step toward the successful treatment of advanced disease.

COX\(^3\), also referred to as prostaglandin endoperoxide synthase, is a key enzyme in the conversion of arachidonic acid to prostaglandins and other eicosanoids. Two isoforms of COX have been identified. COX-1 is expressed constitutively in a number of cell types, whereas COX-2 is inducible by a variety of factors, which include cytokines, growth factors, and tumor promoters (5). COX-2 was identified initially as a member of early growth response gene group (6, 7). Forced expression of COX-2 in rat intestinal cells results in an increased level of bcl-2 protein and resistance of the cells to apoptosis, suggesting a potential strategy for cancer prevention and treatment by modulating COX-2 activity (8). In addition, prostaglandin E\(_2\), a major product of COX-2, has been shown to inhibit programmed cell death and up-regulate bcl-2 expression in a human colon cancer cell line (9). Clinically, continuous use of aspirin and other nonsteroidal anti-inflammatory drugs has been shown to reduce the risk of colorectal cancer in humans by 40–50% (10). Moreover, recent reports reveal that treatment with highly selective COX-2 inhibitors results in a reduction in the size and number of premalignant and malignant colonic lesions in several animal models (11, 12).

LNCaP is an androgen-sensitive human prostate cancer cell line, which has been reported to exhibit a high constitutive level of COX-2 mRNA (13). In this report, we demonstrate that NS398, a selective COX-2 inhibitor (14), induces apoptosis in LNCaP cells but not in human fetal prostate fibroblasts. The NS398-induced apoptosis in LNCaP cells is associated with down-regulation of bcl-2 protein expression.

Materials and Methods

Cell Culture. The LNCaP cell line was purchased from American Type Culture Collection (Rockville, MD). Human fetal prostate fibroblasts were isolated from 18- to 24-week-old gestation fetuses at the time of pregnancy interruption, as described previously (15). The established fibroblast cell line was confirmed by positive immunohistochemical staining for vimentin and negative staining for smooth muscle markers. Both cell lines were routinely maintained in RPMI 1640 containing 10% FBS (Life Technologies, Inc., Grand Island, NY).

Cell Viability Assay. LNCaP cells and human fetal prostate fibroblasts were plated at 1 \( \times \) 10\(^6\) cells/well in six-well cluster dishes with 2 ml of 10% FBS-supplemented medium for 24 h. At day 0, cells were washed with PBS, and the medium was changed to serum- and phenol red-free medium containing 0.1% BSA. Incubations were continued with or without increasing concentrations of NS398 (Cayman Chemicals, Ann Arbor, MI) for 5 days with refeeding after 3 days. For the time course experiments, cells were incubated with or without 100 \( \mu \)M NS398 and harvested at various intervals. Dead cells were removed by gentle washing with ice-cold PBS, and the number of living cells after treatment was determined by counting in a hemacytometer or by the trypan blue dye exclusion assay.

Nuclear Staining Assay. After treatment of LNCaP cells and human fetal prostate fibroblasts with 100 \( \mu \)M NS398 for 3 days, cells were washed with cold PBS, trypsinized, and harvested by centrifugation. Apoptotic cells were detected by 4,6-diamidino-2-phenylindole (DAPI, Oncor, Gaithersburg, MD) staining. Briefly, cells were washed once with ice-cold PBS before being fixed in a solution of methanol/acetone (3:1) for 30 min. Fixed cells were placed on slides and were stained with 1 \( \mu \)g/ml of DAPI for 15 min. The nuclear morphology of cells was observed by fluorescence microscopy.
Qualitative and Quantitative DNA Fragmentation Analysis. Qualitative DNA gel fragmentation assay was performed as described by Moore and Matlashewski (16). Briefly, subconfluent cells were cultured in RPMI 1640 containing 10% FBS with or without NS398 (100 μM) for various times. Cells were washed with cold PBS and lysed in lysis buffer [10 mM Tris (pH 7.4), 5 mM EDTA, and 1% Triton X-100] for 20 min on ice. Microcentrifugation was performed at 11,000 × g for 20 min to separate the nuclear DNA precipitate from the fragmented DNA present in the supernatant. The supernatant was treated with 50 μg/ml of RNase A at 37°C for 1 h, and then proteinase K was added at 0.1 mg/ml for another hour. After phenol-chloroform extraction, DNA from the supernatant was precipitated by ethanol and resuspended in 100 μl of TE buffer [10 mM Tris (pH 8.0) and 1 mM EDTA]. The equal amounts of DNA samples (20 μg) were electrophoresed on a 1.2% agarose gel and visualized by ethidium bromide staining. Quantification of DNA fragmentation was accomplished using the diphenylamine reagent as previously described (17).

TUNEL Assay for Apopotic Cells. LNCaP cells and human fetal prostate fibroblasts were incubated in medium containing 10% FBS with or without NS398 for 3 days. Cells were washed with ice-cold PBS and harvested by centrifugation. Apoptotic cells were detected using the TUNEL method with the ApopTag in situ apoptotic detection kit according to the manufacturer’s instruction (Oncor). The labeled cells were examined using a fluorescent microscope.

Immunoblotting. Cells cultured under the desired conditions were lysed as described previously (18). Briefly, cells were rinsed twice and scraped with 1.5 ml of ice-cold Ca²⁺- and Mg²⁺-free PBS containing 4 mM iodoacetate. After centrifugation, the pellets were resuspended in 50 μl of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPs) extraction solution [10 mM CHAPs, 2 mM EDTA (pH 8.0), and 4 mM iodoacetate in PBS] with protease inhibitors. The samples were then incubated for 30 min on ice and centrifuged at 15,000 × g for 10 min. The supernatants were electrophoresed on a 12% SDS-polyacrylamide gel, electrophoretically transferred to a polyvinylidene difluoride membrane (DuPont, Wilmington, DE), and incubated with an anti-bcl-2 monoclonal antibody (Oncogene Research Products, Cambridge, MA) for 2 h. Secondary horseradish peroxidase-linked donkey antiamouse IgG was used. Filters were developed by the enhanced chemiluminescence system (Amersham, Arlington Heights, IL). Relative protein expression was then quantified with a densitometer (Molecular Dynamics, Sunnyvale, CA).

Results

Effects of NS398 on LNCaP Cell and Human Fetal Prostate Fibroblast Viability. Initial experiments compared the effects of NS398 on cell viability in LNCaP cells and human fetal prostate fibroblasts. As shown in Fig. 1A, treatment with 100 μM NS398 significantly decreased the viability of LNCaP cells after 2 days (68% of control). LNCaP cell survival was 48 and 8% of control after 3 and 5 days treatment with NS398, respectively. The dose dependence of the NS398 effect was next characterized. As shown in Fig. 1B, a modest decline in viable tumor cells was discernible after treatment with 1 μM NS398 for 5 days. Higher concentrations of the inhibitor further decreased cell survival. In contrast, the viability of human fetal prostate fibroblasts was unaffected by NS398. In separate experiments, cell death was also assayed by trypan blue exclusion, and similar results were obtained (data not shown). These results indicated that NS398 decreased LNCaP cell viability in a time- and dose-dependent fashion, with no discernible effect on human fetal prostate fibroblasts.

NS398-induced Cell Death Caused by Apoptosis. One of mechanisms responsible for the NS398-induced cell death in LNCaP cells could be apoptosis. To test this hypothesis, we first examined the nuclear morphology of dying cells using a fluorescent DNA-binding agent, DAPI. As shown in Fig. 2A, LNCaP cells cultured in the absence of NS398 showed intact nuclei. In contrast, within 3 days of treatment with 100 μM NS398, LNCaP cells clearly exhibited condensed and fragmented nuclei indicative of apoptotic cell death. No altered nuclear morphology was evidenced in human fetal prostate fibroblasts with or without 100 μM NS398 for 3 days.

To confirm the apoptosis induced by NS398, the free 3' ends generated by apoptotic DNA cleavage were also detected immunohistochemically by TUNEL assay, in which the nonapoptotic cells stained red while apoptotic cells stained yellow or green. As demonstrated in Fig. 2B, both human fetal prostate fibroblasts with or without NS398 and control LNCaP cells were red, whereas LNCaP cells treated with 100 μM NS398 for 3 days stained yellow and green, indicating apoptosis.

NS398-induced apoptosis was further examined with the DNA fragmentation assay, which is considered a hallmark of apoptosis. As shown in Fig. 3, upper panel, NS398-treated LNCaP cells produced a smear of different sizes of DNA fragments and a distinct oligosomal ladder, a typical characteristic of cells undergoing apoptosis. In contrast, neither control LNCaP cells nor human fetal prostate fibroblasts showed detectable DNA fragments. The quantitative assay for DNA fragmentation is shown in Fig. 3, lower panel. DNA fragmentation in LNCaP cells was detectable within 24 h after treatment and continuously enhanced in the presence of 100 μM NS398. NS398 Down-Regulated bcl-2 Protein Expression. bcl-2 has been identified as an apoptosis-suppressing oncoprotein (19). To evaluate the molecular mechanisms involved in the NS398-induced LNCaP cell apoptosis, the effect of NS398 on bcl-2 protein expression...
Fig. 2. Induction of apoptosis by NS398. A. Nuclear condensation and fragmentation. Human fetal prostate fibroblasts (a and b) and LNCaP cells (c and d) were treated either with vehicle as control (a and c) or with 100 μM NS398 (b and d) for 3 days. Cells were harvested and washed with ice-cold PBS, followed by fixation in methanol:acetic acid (3:1) for 30 min. The fixed cells were incubated with 1 μg/ml of DAPI, and nuclear staining was examined by a fluorescent microscope. Arrows, apoptotic cells with condensed or fragmented nuclei. B. Detection of apoptotic cells by the TUNEL assay. Human fetal prostate fibroblasts (a and b) and LNCaP cells (c and d) were treated either with vehicle as control (a and c) or with 100 μM NS398 (b and d) for 3 days. Cells were harvested and washed with ice-cold PBS, trypsinized, and harvested with centrifugation. Apoptotic cells were detected by ApopTag in situ apoptosis detection kit and were examined by a fluorescent microscope. Data shown are representative of two independent experiments.
COX-2 INHIBITOR INDUCES LNCaP CELL APOPTOSIS

high basal levels of both COX-2 mRNA (13) and protein, whereas basal COX-2 expression was barely detectable in human fetal prostate fibroblasts. The present study demonstrates that NS398, a selective COX-2 inhibitor, induces apoptosis in LNCaP cells but not in human fetal prostate fibroblasts, suggesting the existence of distinct signal pathways in the regulation of apoptosis in LNCaP cells in which COX-2 activity is involved.

bcl-2 expression has been linked to the inhibition of apoptosis in several different cell types (19, 24). Extended studies have revealed that bcl-2 prevents the release of apoptosis-inducing factor (25) and cytochrome C from mitochondria (26), which is assumed to be a key event during apoptosis. In addition, phosphorylation of bcl-2 has been reported to inactivate the protein (20). Our data indicate that NS398 treatment of LNCaP cells down-regulates the expression of the active bcl-2 protein while it up-regulates the expression of the inactive, phosphorylated protein. These changes in the levels of the active/inactive bcl-2 protein occurred prior to DNA fragmentation, suggesting a role for bcl-2 in the mediating, or triggering, of apoptosis induced by NS398 in LNCaP cells.

A role for COX-2 in cancer was first reported by Tsujii and DuBois (8). Since then, dramatic progress has been made. Several selective

was examined. As demonstrated in Fig. 4, Western blot analysis revealed that LNCaP cells expressed a basal level of bcl-2 (M, 26,000 protein). The LNCaP cells also expressed a slower migrating band with a molecular weight of M, 35,000, previously identified as a tyrosine phosphorylated bcl-2 protein (20). With the addition of NS398, the bcl-2 (M, 26,000 protein) level declined within 4 h, and that decreased further over a 24-h period after NS398 treatment. Interestingly, the M, 35,000 protein corresponding to the inactive, phosphorylated bcl-2 was up-regulated, with a peak increase noted 16 h after NS398 addition.

Discussion

COX-2 is an inducible enzyme in most tissues and cell types. However, it is constitutively expressed in a number of human cancer cell lines, including prostate cancer cells (13, 18, 21, 22). The persistent presence of COX-2 may result in increased expression of genes normally induced only transiently during passage through the cell cycle (8, 23). The LNCaP human prostate cancer cell line exhibits
COX-2 inhibitors have been tested for their possible utility as chemopreventive agents in colon cancer (11, 12). In addition, a recent report demonstrated the involvement of COX-2 in the regulation of tumor angiogenesis (27), suggesting a novel mechanism whereby COX-2 inhibitors inhibit tumor growth. However, studies on the role of COX-2 in other types of human cancer are limited. This report, to our best knowledge, is the first to demonstrate the induction of apoptosis by a selective COX-2 inhibitor in human prostate cancer cells. The recent development of a second generation of highly selective COX-2 inhibitors (28) holds promise for future therapies that specifically target cancer cells. Our findings suggest that additional studies that determine the efficacy of selective COX-2 inhibitors in the treatment of human prostate cancer are warranted.

References
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