Non-steroidal anti-inflammatory drugs inhibit matrix metalloproteinase-2 expression via repression of transcription in lung cancer cells

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Abstract Recent studies show that up-regulation of cyclooxygenase-2 (COX-2) in human cancer cells induces activation of matrix metalloproteinases (MMPs) and increase of metastatic potential. In this study, we investigate the effect of a COX-2 selective inhibitor, NS398, on the expression and enzymatic activity of MMPs in human lung cancer cells. We found that NS398 inhibited MMP-2, not MMP-9, mRNA expression. NS398 also reduced the amount of MMP-2, not MMP-9, released into the medium. Additionally, this COX-2 inhibitor attenuated the degrading activity of MMP-2 as demonstrated by gelatin zymography. Investigation of cellular MMP-2 by Western blotting indicated that synthesis and processing of MMP-2 was significantly suppressed by NS398. We performed promoter activity assay to address whether NS398 might affect MMP-2 gene transcription. Our results indicated that NS398 directly inhibited MMP-2 promoter activity. However, the inhibitory effect of NS398 is not fully dependent on inhibition of COX-2 because a high concentration of NS398 was needed to suppress MMP-2 expression and addition of prostaglandin E_2 only partially reversed the action of NS398. Moreover, a non-selective COX inhibitor indomethacin also suppressed the expression of MMP-2. Taken together, these results indicate that non-steroidal anti-inflammatory drugs suppress MMP-2 expression via repression of transcription and support the notion that COX inhibitors may be useful in inhibition and/or prevention of metastasis.

Key words: Matrix metalloproteinase-2; NS398; Cyclooxygenase-2; Promoter; Lung cancer

1. Introduction

Prostaglandins (PGs) play a critical role in various physiological and pathophysiological processes, including blood clotting, kidney function, wound healing, cardiovascular disease and inflammation [1,2]. Recent works show that cyclooxygenase (COX) is the rate-limiting enzymes involved in conversion of arachidonic acid to PGs. Two isoforms of COX, COX-1 and COX-2, have been identified. COX-1 is expressed constitutively in various tissues [3]. On the contrary, COX-2 expression is induced by mitogens [4,5], cytokines [6,7] or tumor promoters [8]. Recent studies have demonstrated that increased expression of COX-2 is frequently found in human cancers including breast, lung, gastric, esophageal, pancreatic and colon cancer [9–14]. In addition, lines of evidence have indicated that non-steroidal anti-inflammatory drugs (NSAIDs), potent inhibitors of COXs, exert chemopreventive effect on the development of these cancers [15].

Recent studies indicated that overexpression of COX-2 may induce tumor metastasis [16]. Interestingly, a positive correlation between COX-2 expression and lymphatic invasion has been reported in colon and gastric cancer [17,18]. However, the function of COX-2 in tumor metastasis is unclear. The process of tumor invasion by cancer cells involves degradation of the underlying basement membrane, which is largely made up of type-IV collagen. The matrix metalloproteinases (MMPs) are a growing family of zinc-dependent endopeptidases that selectively degrade components of the extracellular matrix. The MMP family can be subgrouped into at least four types based on substrate specificity and sequence characteristic. There are the interstitial collagenases, the stromelysins, the gelatinases and the membrane-type MMPs [19]. Among the MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) show substrate specificity toward type IV and V collagen and a number of studies have demonstrated a strong correlation between gelatinase expression and metastatic potential [20,21]. Additionally, recent works demonstrated that enforced expression of COX-2 in cells induced activation of MMP-2 and MMP-9 [22,23]. In this study, we investigate the effect of selective or non-selective COX-2 inhibitors on the expression and activation of MMPs in human lung cancer cells and our results demonstrate that these inhibitors directly inhibit MMP-2 expression via repression of transcription.

2. Materials and methods

2.1. Cell culture and chemicals

A549 human lung cancer cells were cultured in Dulbecco's modified Eagle's medium and F12 nutrition mixture (DMEM/F12) supplemented with 10% heated–inactivated fetal calf serum (FCS) and antibiotics. PGE_2 and type-A porcine skin gelatin were obtained from Sigma (St. Louis, MO, USA). NS398 was purchased from Biomol (Polymouth Meeting, PA, USA). Monoclonal antibodies against human MMP-2 and MMP-9 were obtained from Oncogene Research Product (Boston, MA, USA). Luciferase and β-galactosidase enzyme assay systems were from Promega (Madison, WI, USA).

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Abbreviations: NSAID, non-steroidal anti-inflammatory drug; PG, prostaglandin; COX, cyclooxygenase; MMP, matrix metalloproteinase

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2.2. RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from control or drug-treated cells using a RNeasy mini kit from Qiagen (Santa Clarita, CA, USA). 1 μg of total RNA was reverse-transcribed to cDNA by using the OneStep RT-PCR kit (Qiagen) according to the manufacturer's protocol. Each PCR reaction was performed in 50 μl of a reaction mix, containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.4 mM dNTP, 600 μM primers and three units of HotStarTaq DNA polymerase. 5 μl of the reverse-transcribed cDNA samples were added to the reaction mix and amplified for 30 cycles: denaturation at 94°C for 45 s, annealing at 60°C for 45 s, extension at 72°C for 2 min and final extension at 72°C for 10 min. A constitutively expressed gene, glyceraldehyde-3-phosphate dehydrogenase (G3PDH), was used as an internal control to check the efficiency of cDNA synthesis and PCR amplification. The primer sequence and PCR product size for MMP-2 was 5′-GTGCTGAAGGACACACTAAAGAAGA-3′ and 5′-TTGCCATCCTTCTCAAAGTTGTAG-3′; 580 bp. The primer sequence and PCR product size for MMP-9 was 5′-GCCACTTGTCGGCGATAAGG-3′ and 5′-CACTGTCCACCCCTCAGAGC-3′; 243 bp. Amplified cDNA products were run on 2% agarose gels, stained with ethidium bromide and visualized under UV light.

2.3. Western blot analysis

Conditioned medium was concentrated 25-fold and equal amounts, based on cell numbers determined at the time of harvest, of supernatant were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis was performed as described previously [24]. For analysis of cellular MMP-2 protein, cells were treated with vehicle or NS398 (100 μM) for 24 h. Cellular proteins were extracted and protein concentrations were determined by a BCA assay kit. Protein level and processing of MMP-2 was investigated by probing the nitrocellulose membranes with anti-MMP-2 monoclonal antibody.

2.4. Zymographic assays for MMP-2

Conditioned medium was concentrated 25-fold and equal amounts, based on cell numbers determined at the time of harvest, of supernatant were mixed with 5×gel loading buffer (0.5 M Tris–HCl, pH 6.8, 10% SDS, 50% glycerol and 0.5% bromophenol blue). Samples were separated in a 7.5% SDS–PAGE gel containing 1 mg/ml gelatin. The gels were washed for 1 h at room temperature in a solution containing 2.5% (v/v) Triton X-100 and subsequently incubated with a reaction buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$ and 0.5 mM $ZnCl_2$) at 37°C overnight. The gels were stained for 1 h with staining solution (0.1% Coomassie brilliant blue, 30% methanol and 10% acetic acid) and destained in the same solution without Coomassie brilliant blue and were dried directly between dialysis membranes.

2.5. Promoter activity assays

Effect of PGE_2, NS398 and indomethacin on MMP-2 promoter activity was analyzed as described previously [24]. Cells were plated onto six-well plates at a density of 10^5 cells/well and grown overnight. Cells were cotransfected with 2 μg of MMP-2 promoter–luciferase reporter construct (kindly provided by Dr. Benveniste) and 2 μg of β-galactosidase reporter plasmid by the LipofecAMINE method. Cells were cultured in 10% FCS medium and incubated with vehicle or drugs for 24 h. Luciferase activity and β-galactosidase activity were assayed by using the luciferase and β-galactosidase enzyme assay system (Promega). Luciferase activity was normalized with the β-galactosidase activity in cell lysate and calculated as an average of three independent experiments.

3. Results

We first address whether COX-2 inhibitor NS398 may affect the expression and activation of MMP-2 and MMP-9. Control or drug-treated cells were harvested and the mRNA level of MMP-2 and MMP-9 was analyzed by RT-PCR. As shown in Fig. 1, our results indicated that NS398 inhibited MMP-2 expression in a dose-dependent manner. Conversely,

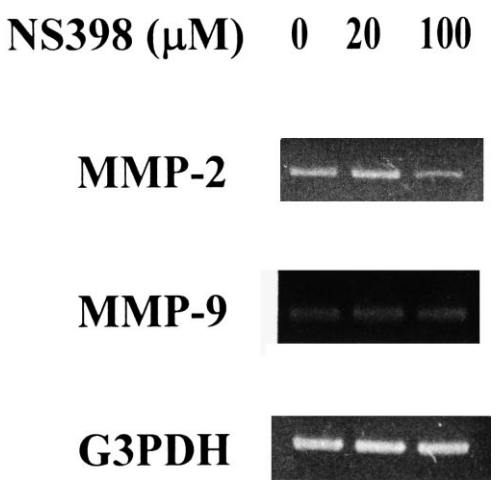

Fig. 1. NS398 inhibits MMP-2, not MMP-9, expression in lung cancer cells. A549 cells were cultured in 10% FCS medium containing vehicle or different concentrations of NS398 for 24 h. Total RNA was isolated and mRNA level of MMP-2, MMP-9 and G3PDH were examined by RT-PCR analysis. Amplified cDNA products were run on 2% agarose gels, stained with ethidium bromide and visualized under UV light.

NS398 could not regulate the expression of MMP-9. The efficiency of cDNA synthesis and PCR amplification was checked by using a pair of primers designed to amplify G3PDH gene fragment. A single band with predicated size (283 bp) was detected in all samples and similar levels of this gene fragment were observed in all samples. We next investigated the amounts of MMP-2 secreted into the culture medium by Western blot analysis. Our data confirmed the results of RT-PCR and showed that NS398 reduced the amounts of active MMP-2 protein (66 kDa), but not active MMP-9 (83 kDa), in the conditioned medium (Fig. 2). We also investigated the expression and processing of MMP-2 in cells. As demonstrated in Fig. 3A, synthesis of MMP-2 precursor protein (72 kDa) and processing of this precursor to the active form of MMP-2 were dramatically inhibited by NS398. In addition, NS398 effectively suppressed MMP-2 activity in the culture medium as demonstrated by zymographic assays (Fig. 3B).

Because NS938 down-regulated MMP-2 mRNA in lung cancer cells, we proposed that NS398 might directly affect MMP-2 expression by inhibiting gene transcription. We addressed this question by analyzing the effect of NS398 on MMP-2 promoter activity. Cells were transfected with MMP-2 promoter–luciferase construct, treated with different concentrations of NS398 for 24 h. and luciferase activity was determined. As shown in Fig. 4, our results demonstrated that NS398 suppressed MMP-2 promoter activity in a dose-dependent manner. Additionally, we found that a non-selective COX inhibitor indomethacin also potently inhibited MMP-2 promoter activity. Because PGE_2 is the enzymatic product of COX, we tested whether PGE_2 might reverse the inhibitory effect of NS398 on MMP-2 promoter activity. Our results demonstrated that suppression of MMP-2 promoter activity by NS398 was partially reversed by PGE_2 in lung cancer cells (Fig. 5). These data suggest that NSAIDs may directly interfere MMP-2 expression and the inhibitory action of these drugs may not be totally dependent on their ability to inhibit COXs.

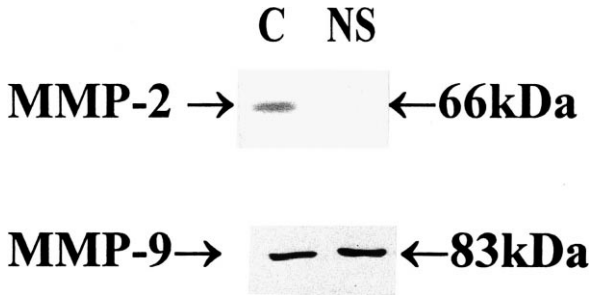

Fig. 2. Protein level of MMP-2, but not MMP-9, in the conditioned medium was reduced by NS398. A549 Cells were cultured in serum-free medium containing vehicle or 100 μM NS398 for 24 h. Conditioned medium was concentrated 25-fold and equal amounts, based on cell numbers determined at the time of harvest, of supernatant were separated by SDS–PAGE. MMP-2 and MMP-9 protein level were determined by Western blot analysis.

4. Discussion

Lines of evidence have demonstrated that COX inhibitors exhibit potent anti-angiogenic and anti-metastatic activity in vitro and in vivo [25,26]. However, the molecular mechanism of these actions is not clear. Recent studies indicate that MMPs play important roles in the development of metastasis and angiogenesis. Because MMP-2 and -9 are the principle enzymes that involved in the degradation of type IV collagen in the extracellular matrix, it has been proposed that these two MMPs are key players in cancer invasion and metastasis. Indeed, a positive correlation between the expression of MMP-2 and -9 and tumor metastasis has been observed in human lung cancer [27,28].

Two crucial findings in our study should be noted. First, this work is the first one to demonstrate that COX inhibitors

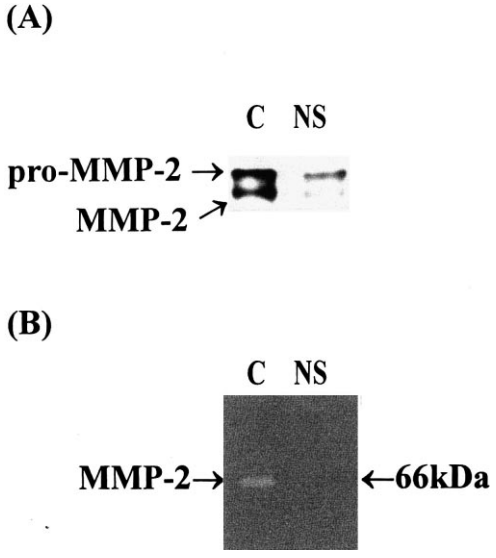

Fig. 3. NS398 inhibits MMP-2 protein synthesis, processing and enzymatic activity. A: Cells were treated with vehicle or 100 μM NS398 for 24 h. Cellular proteins were extracted and synthesis and processing of MMP-2 was investigated by Western blot analysis. B: Cells were cultured in serum-free medium containing vehicle or 100 μM NS398 for 24 h. Conditioned medium was concentrated 25-fold and equal amounts, based on cell numbers determined at the time of harvest, of supernatant were subjected to zymographic assay as described in Section 2.

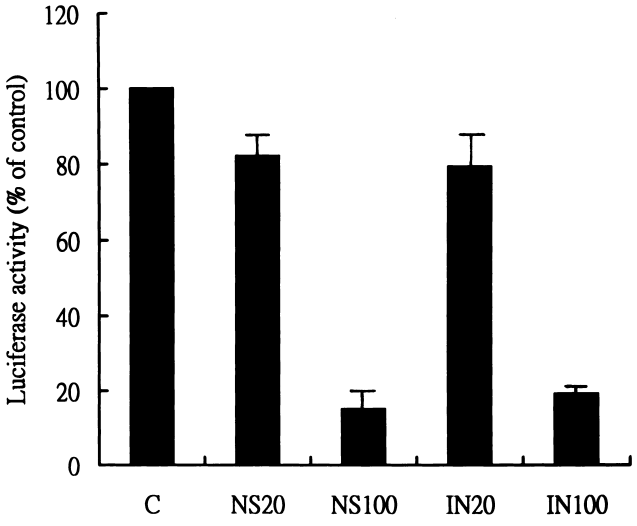

Fig. 4. COX inhibitor NS398 and indomethacin suppress MMP-2 promoter activity in a dose-dependent manner. A549 cells were co-transfected with 2 μg of MMP-2 promoter–luciferase construct and 2 μg of β-galactosidase reporter plasmid by the LipofecAMINE method. Cells were incubated with vehicle or different concentrations (20 or 100 μM) of NS398 (NS) or indomethacin (IN) for 24 h. Luciferase activity was determined, normalized with β-galactosidase activity and expressed as the percentage of that of vehicle-treated cells.

may directly inhibit MMP-2 expression via repression of gene transcription. A number of potential regulatory elements including Sp1, Ap-1, Ap-2, CREB, C/EBP, Ets-1, c-Myc, and PEA3 binding sites have been identified in the human MMP-2 promoter [29]. We are now investigating the signaling pathway by which these inhibitors inhibit MMP-2 expression and mapping the critical regions in the MMP-2 promoter that mediated this inhibitory effect. Second, because the concentration of NS398 needed to suppress the expression of MMP-

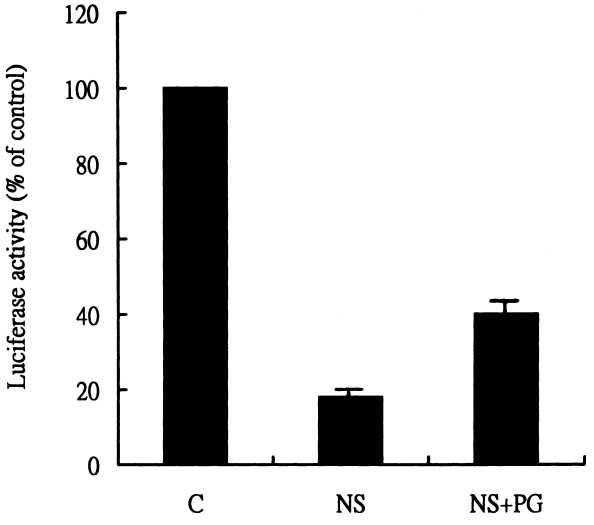

Fig. 5. PGE_2 partially reverses NS398-induced inhibition of MMP-2 promoter activity. A549 cells were cotransfected with 2 μg of MMP-2 promoter–luciferase construct and 2 μg of β-galactosidase reporter plasmid and incubated with vehicle (C) or 100 μM NS398 (NS) or 100 μM NS398+10 μM PGE_2 (NS+PG) for 24 h. Luciferase activity was determined, and normalized with β-galactosidase activity and expressed as the percentage of that of vehicle-treated cells.

2 is much higher than the concentration of NS398 needed to block the enzymatic activity of COX-2, we speculate that the effect of NS398 on MMP-2 expression is not totally dependent on inhibition of COX-2 activity. Indeed, a non-selective COX inhibitor indomethacin also exerts similar inhibition of MMP-2 expression. Furthermore, addition of a high dose of PGE_2 only partially reversed the action of NS398. Interestingly, a very recent study demonstrated that aspirin, another non-selective COX inhibitor, may effectively inhibit MMP-2 production and migration of hepatoma cells [30]. However, the authors did not elucidate the mechanism of this action. Our data provide a molecular basis for the anti-angiogenic and anti-metastatic action of NSAIDs.

Taken together, we test the hypothesis that COX inhibitors may suppress the activity of MMPs to inhibit tumor angiogenesis and metastasis in this work. Our results support this notion and suggest that NSAIDs may be useful for the treatment or prevention of metastasis of lung cancer.

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