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Expression of MMP2 and MMP9 (Gelatinases A and B) in Human Colon Cancer Cells

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Abstract Matrix metalloproteinases (MMPs) are a family of zinc-dependent neutral endopeptidases, collectively capable of degrading essentially all matrix components. Elevated levels of distinct MMPs are detected in tumor tissue or serum of patients with advanced cancer, and they are the major prognostic indicators in cancer. Inhibition of MMPs has been explored as a therapeutic goal for almost two decades. Nitric oxide (NO), a free radical plays an important role in signaling pathways in regulation of MMP expression. In the present study, we demonstrated the role of exogenous NO levels in the regulation of MMP2 and MMP9 (gelatinases A and B) in colon cancer cell line WiDr and its inhibition with emodin (a naturally occurring anthraquinone).

Keywords Matrix metalloproteinase · Colon cancer · Emodin · Sodium nitroprusside · Nitric oxide

Abbreviations

MMP	Matrix metalloproteinases
NO	Nitric oxide
ECM	Extracellular matrix
NOs	Nitric oxide synthases
DMEM	Dulbecco's modified eagle medium
PBS	Phosphate-buffered saline
EDTA	Ethylenediaminetetra acetate
SNP	Sodium nitroprusside
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay

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RT-PCR	Reverse transcriptase-PCR
MoMLV	Molony murine leukemia virus
TAE	Tris acetate-EDTA buffer
MMPI	Matrix metalloproteinases inhibitors

Introduction

Colon cancer is the third most common form of cancer and the second leading cause of death among cancer patients. Despite major advances in the diagnosis and treatment of this disease, its mortality has remained unchanged during the last 20 years. Cells from malignant tumors move and continue to duplicate forming new tumor, a process called metastasis [1]. Tumor invasion and metastasis are controlled by various matrix metalloproteinases (MMPs). MMPs, a family of zinc-dependent endopeptidases involved in the degradation of ECM, play a relevant role in tumor progression by providing a permissive micro-environment for tumor invasion and metastasis [2]. Major MMPs, including gelatinase A (MMP2) and gelatinase B (MMP9), have a key role in the proteolytic cascade-leading ECM cleavage during metastasis in colon carcinoma [3]. Nitric oxide (NO) is a free radical generated by a family of nitric oxide synthases (NOs). NO has important role in the regulation of tumor angiogenesis and metastasis [4]. NO promotes de novo tumorigenesis when associated with chronic inflammation, angiogenesis, and the growth of established solid tumors, whereas it mediates antitumor-cell activity against hematogeneously disseminating tumor cells [5]. Emodin(1,3,8-tri hydroxy-6-methyl-9,10-anthraquinone), a naturally occurring anthraquinone present in roots and barks of Chinese herbs such as *Polygonum cuspidatum* and *Rheum officinalae* having anti-inflammatory and antitumor activity [6]. From ancient times, the compound has been used as an active component in herbal extracts for medical treatment. Recently, several scientific studies of its biological activity have been performed regarding the anti proliferative effect of emodin on several human cancers such as multiple myeloma and lung squamous cell carcinoma [7, 8]. The presence of hydroxyl groups in position 1, 3, and 8 of the aromatic ring system is essential for the purgative action of the compound [9]. There are not much scientific reports validating the role of these compounds in modulating the regulatory molecules involved in metastasis. Understanding the mechanism of inhibition of key regulatory molecules which is responsible for metastasis may provide valuable information for the possible use of these compounds in cancer chemotherapy. In the present study, I took emodin to evaluate its anti-metastatic ability. The aim of the present study was to investigate the expression of MMP2 and MMP9 (gelatinases A and B) in human colon cancer cell line WiDr.

Materials and Methods

Cell Culture

Cells of human colonic adenocarcinoma cell line WiDr was grown at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were cultured in DMEM medium, which is supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin. When the cells were grown to confluency, the medium was removed and washed once with PBS, 0.25% Trypsin-EDTA solution was added and cells were dispersed gently by a pipette. A known number of cells were dispensed in to new microtitre plates for further experiments.

MTT-Cell Proliferation Assay

The present study evaluated the role of exogenous nitric oxide in the regulation of matrix metalloproteinases and its inhibition with emodin in colon cancer cell line. For this, we carried out a cell viability assay (MTT assay) for determining a nontoxic concentration of sodium nitroprusside (SNP, an exogenous NO donor) and the concentrations of SNP were selected for further experiments. MTT assay [10] is based on the metabolic activities of the viable cells. In this assay, a tetrazolium salt MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) was used. Briefly, WiDr cells were seeded in to a 96-well microtitre plate. Exogenous NO donor SNP and drug emodin were added in different concentrations in quadruplicate (62.5 to 500 μM for SNP and 6.25 to 50 μM for emodin). Six wells contained cells in drug-free medium to determine the control cell survival and the percentage of live cells after culture. Stock concentrations of drug were prepared in DMSO, and diluted concentrations were made in the medium added. The cells were incubated for 48 h at 37 °C. A fresh medium was added along with 20 μl MTT to each well. The plates were kept for 2 h and 30 min incubation in dark. The formazan crystals formed were solubilized with acidified isopropanol. The plates were kept for 10 min to solubilize the crystals. The color developed was quantitated with an ELIZA plate reader at 570 nm.

Reverse Transcriptase-PCR

Expression of MMP gene was detected using RT-PCR by Trizol method [11]. The cells were incubated with and without 300 μM SNP and 6.25 μM emodin for 24 h and then lysed by adding 1 ml TRIzol reagent. Three hundred microliters of chloroform was added, centrifuged at 10,000 rpm for 15 min at 4 °C. To precipitate total RNA from the aqueoua phase, 600 μl of isopropanol was added and centrifuged at 10,000 rpm for 15 min at 4 °C. The pellet was washed with 70% ethanol, centrifuged, and dried. For synthesizing cDNA, the RNA was incubated with random hexamer. MoMLV reverse transcriptase was used for the synthesis of cDNA strand. The cDNA synthesis was performed at 37 °C for 90 min. The resulting cDNA were subjected to PCR analysis using specific primer sets. PCR was done in 18 μl consisting of 1.5 μg cDNA, 2 μl 10× PCR buffer, 1.5 mM $Mgcl_2$, 1 mM dNTP, 1.25 U Taq DNA polymerase, 5 pmol forward primer, 5 pmol reverse primer, and 12.35 μl water. The primers for MMP2 were TCTTCAAGGACCGGTTCATTTG and GATGCTTCCAAACTTCACGCTC, and MMP9 were CACTGTCCACCCCTCAGAGC and GCCACTTGTCGGCGATAAGG. β-actin was used as an internal control. The primers for β-actin were TGACGGGGTCACCCACACTGTGCCCATCT and GAAGTAG TAAGTGGGAACCGTGT. Amplification was done for 40 cycles with following conditions; denaturation at 95 °C for 30 s, annealing at 50 °C for MMP2, 62 °C for MMP9, and 57 °C for β-actin, extension at 72 °C for 2 min was also given.

Agarose Gel Electrophoresis

Amplified PCR products were run on agarose gel electrophoresis; 0.15 g of agarose was dissolved in 15 ml 1× buffer and melted completely. One microliter of Ethidium bromide was added and allowed to polymerize. The template carrying gel was placed in the electrophoresis tank and filled with 1× TAE buffer. The samples mixed with equal volume of gel loading buffer, and about 4 μl of samples were loaded in to appropriate wells. Electrophoresis was carried out, and the DNA bands were visualized.

Gelatin Zymography

The protein expression of MMP2 and MMP9 was detected by gelatin zymography [12]. Since gelatinases are secreted proteases, the cell culture supernatant was taken for this assay. Cells were grown in 100 mm plates and treated with and without 300 μM SNP, 300 μM SNP, and 6.25 μM emodin for 24 h. After incubation, cell supernatant was taken, filtered, and centrifuged at 5,000 rpm for 30 min at 15 °C; 7.5 μl of the concentrated sample was mixed with equal amount of 2× non-reducing sample buffer and electrophoresed on 10% SDS–PAGE copolymerized with gelatin (2 mg/ml). Following electrophoresis, gel was washed with rinse buffer to wash away SDS, thus allowing the gelatinase to renature. Then, the gel was placed in reaction buffer for 24 h to facilitate gelatin degradation by gelatinase. Then, the gel was incubated for 45 min in Coomassie brilliant blue stain and then destained. The gelatinolytic activities were detected as white bands.

Results

Cytotoxic Profile of SNP on WiDr Cell Line

WiDr cells grown in 96-well plates were treated with 62.5 to 500 μM of SNP, and cell viability was assessed by MTT assay. All the results were expressed as the mean percentage of control ± SD of quadruplicate determinations from the independent experiments. The results showed that SNP at a concentration below 300 μM showed a little effect on cell viability (Fig. 1); hence, we used 300 μM for further experiments to evaluate the role of NO in modulating the expression of MMP2 and MMP9 in the above cell line.

Cytotoxic Profile of Emodin on WiDr Cell Line

The cells grown in 96-well plates were treated with 6.25 to 50 μM of the drug emodin, and cell viability was studied by MTT assay. Emodin at a concentration below 6.25 μM showed a little effect on cell viability, and a concentration above this showed a considerable cytotoxic effect on WiDr cell line (Fig. 2). Therefore, a concentration of 6.25 μM is used for further experiments.

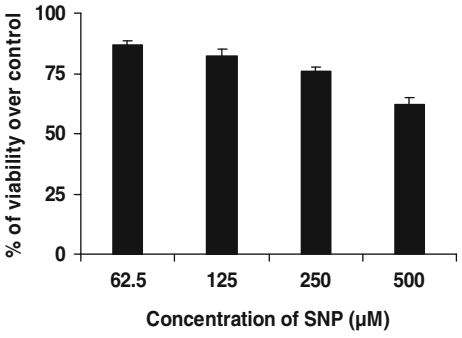

Fig. 1 Cytotoxic profile of SNP on WiDr cell line

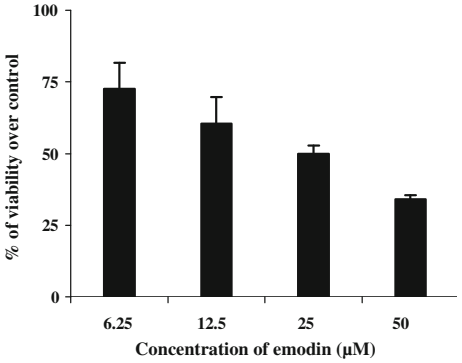

Fig. 2 Cytotoxic profile of Emodin on WiDr cell line

Emodin-Mediated Effect on MMP2 and MMP9 mRNA Levels

Molecular level analysis of MMP2 and MMP9 mRNA levels in SNP and emodin-treated cells using RT-PCR showed a clear correlation of the involvement of these two MMPs in cell migration and the inhibitory action of emodin on it. After treatment with SNP (300 μM) for 24 h, the expression of MMP2 and MMP9 present in the SNP-treated lane showed prominent bands in a concentration dependent manner. This indicatess that the MMP2 and MMP9 mRNA levels in SNP-treated WiDr cell line is significantly increased. Treatment of emodin at a concentration of 6.25 μM inhibited the expression of SNP-induced MMP2 and MMP9 mRNA levels. Thus, the left panel (MMP2) and right panel (MMP9) clearly show that the SNP (300 μM)-treated cell line enhanced the expression of MMP2 and MMP9 mRNA, whereas, the emodin (6.25 μM) treatment inhibited the expression of SNP-induced MMP2 and MMP9 mRNA levels (Fig. 3).

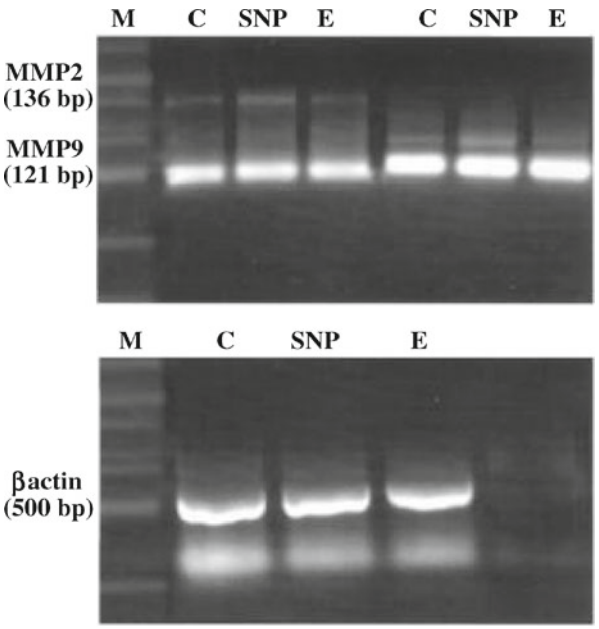

Fig. 3 RT-PCR analysis for detecting the effect of emodin on MMP2 and MMP9 mRNA levels in WiDr cell line

M-Marker, C-Control, SNP-Sodium nitroprusside, E-Emodin

Effect of Emodin on MMP2 and MMP9 Protein Expression

The gelatinolytic bands present in SNP-treated lane showed much clear bands in a concentration-dependent manner compared to the control. This indicates that SNP (300 μM) treatment for a period of 24 h increased MMP2 and MMP9 expression in WiDr cell line compared to that in the untreated cell. Treatment of emodin at a concentration of 6.25 μM decreased SNP-induced protein level of MMP2 and MMP9 (Fig. 4).

Discussion

The present study demonstrated the role of exogenous NO levels in the regulation of MMP2 and MMP9 in a colon cancer cell line (WiDr) and its inhibition with emodin, a naturally occurring anthraquinone. Digestion of ECM encircling the tumor tissue is an essential step in tumor progression especially in cell invasion, migration, and metastasis [2, 3]. Formation and expression of MMPs in tumor cells therefore plays an important role in tumor progression. Initially, we studied the least cytotoxic concentration of SNP and emodin in WiDr cells using cell viability assay. NO released from SNP at higher

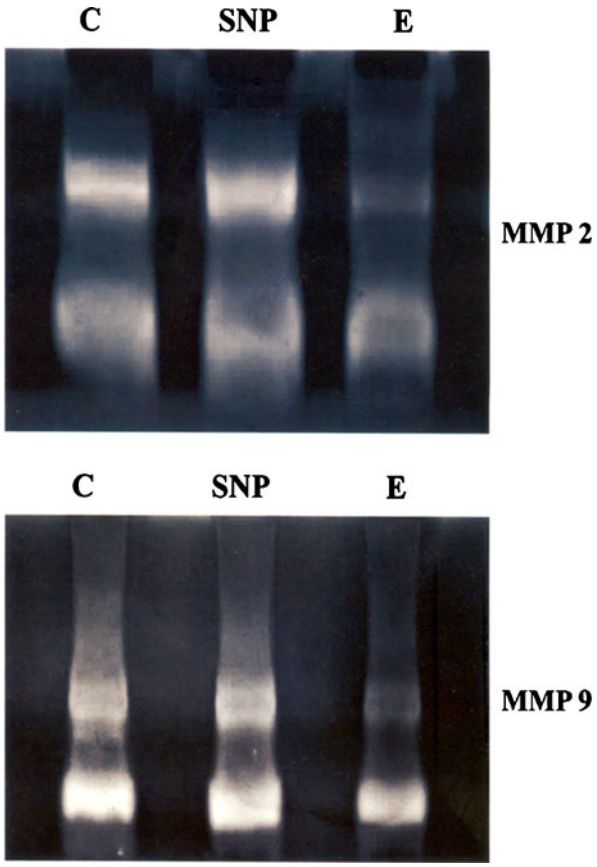

Fig. 4 Zymographic analysis for detecting the effect of emodin on MMP2 and MMP9 expression in WiDr cell line

M-Marker, C-Control, SNP-Sodium nitroprusside, E-Emodin

concentration can kill the cells through Bcl_2, Bax, and P^{53} pathways [13]. We used the derived concentrations for further experiments. Modulation of MMPs is a main target in cancer research. Increased levels of MMP have been demonstrated early in the transition from colon adenoma to adenocarcinoma. Based on the assumption that MMPs were responsible for metastasis, several orally active inhibitors of MMPs (MMPIs) have been developed, and they were found effective in controlling cancer progression in animals [14]. From this study, it is clear that NO levels play an important part in the expression of MMP2 and MMP9 and their inhibition with emodin. The mRNA level analysis indicates the suppression of NO mediated cell migration by emodin. Further, we confirmed our results at the protein level using gelatin zymography in three different conditions such as untreated, SNP-treated, and SNP treated+emodin. Increased gelatinolytic activity of MMP2 and MMP9 in SNP-treated culture supernatant shows the role of NO in increasing prometastatic events leading to the digestion of extracellular matrix components. Decreased gelatinolytic activity in emodin-treated cells shows the involvement in the down regulation of MMP2 and MMP9, thereby inhibiting cell migration. Therefore, NO, MMP2, MMP9, and emodin axis could be a potent therapeutic target in colon cells. Further characterization of emodin-mediated downregulation of MMP2 and MMP9 in colon cancer was important subject for future research. There are numerous reports suggesting the antimetastatic property of emodin in tumor cells. Among the 44 anthraquinones tested against bacterial collagenase in vitro, emodin proved to be the most potent active inhibitor [15]. Therefore, elucidation of critical pathways in metastasis where emodin could exert its inhibitory effects should make it a perfect fit for antitumor therapy.

Conclusion

MMP2 and MMP9 are very important in colon cancer progression. Inhibition of these MMPs by synthetic MMPIs has been considered to be an effective approach to block colon cancer. NO, playing a lead role in signaling pathways, has an important role in colon cancer progression. Emodin, a naturally occurring anthraquinone, suppresses the NO-mediated upregulation of MMP2 and MMP9. Thus, emodin can be targeted as an effective antimetastasis agent in NO-induced tumor progression. Therefore, elucidation of critical pathways in metastasis, where emodin could exert its inhibitory effects, should make it a perfect fit for antitumor therapy.

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