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Quercetin downregulates matrix metalloproteinases 2 and 9 proteins expression in prostate cancer cells (PC-3)

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Abstract

Background: Cancer metastasis, involving multiple processes and various cytophysiological changes, is a primary cause of cancer death and may complicate the clinical management, even lead to death. Quercetin is a flavonoid and widely used as an antioxidant and recent studies have revealed its pleiotropic anticancer and antiproliferative capabilities. Gelatinases A and B (matrixmetalloproteinases 2 and 9) are enzymes known to involve in tumor invasion and metastases. In this study, we observed the precise involvement of quercetin role on these proteinases expression and activity. *Design and methods:* PC-3 cells were treated with quercetin at various concentrations (50 and 100 μM), for 24 h period and then subjected to western blot analysis to investigate the impact of quercetin on matrix metalloproteinase-2 (MMP-2) and 9 (MMP-9) expressions. Conditioned medium and cell lysate of quercetin-treated PC-3 cells were subjected to western blot analysis for proteins expression of MMP-2 and MMP-9. Gelatin zymography was also performed in quercetin treated PC-3 cells. *Results:* The results showed that quercetin treatment decreased the expressions of MMP-2 and MMP-9 in dose-dependent manner. The level of pro-MMP-9 was found to be high in the 100 μM quercetin-treated cell lysate of PC-3 cells, suggesting inhibitory role of quercetin on pro-MMP-9 activation. Gelatin zymography study also showed the decreased activities of MMP-2 and MMP-9 in quercetin treated cells. *Conclusion:* Hence, we speculated that inhibition of metastasis-specific MMPs in cancer cells may be one of the targets for anticancer function of quercetin, and thus provides the molecular basis for the development of quercetin as a novel chemopreventive agent for metastatic prostate cancer. (Mol Cell Biochem **287:** 109–116, 2006)

Key words: prostate cancer, quercetin, matrixmetalloproteinases, metastases, PC-3 cells

Introduction

Tumor invasion and metastasis represent a mutistep process that depends on the activity of many proteins [1]. Proteolytic degradation of the extracellular matrix (ECM) components is a central event of this process. More specifically, the ability to penetrate the basement membrane (BM) is associated with an increased potential for metastases. Indeed, basement membranes are present at key points in the metastatic cascade; escape from the primary tumor in epithelial malignancies, intravasion and extravasation during hematogenous dissemination as well as perineural and muscular invasion require basement membrane breaching [2]. Several classes of proteinases, including serine proteinases, cystiene proteinases and matrix metalloproteinases (MMP's) have been implicated in tumor cell invasion process [3]. Among these

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different proteinases, MMP's appear to be primarily responsible for much of the ECM degradation observed during invasive process. MMP-2 and MMP-9 have been frequently associated with the invasive metastatic potential of tumor cells including prostate cancer [4, 5].

Several epidemiological and laboratory studies have shown that many vegetables, fruits and grains as well as phytochemicals offer significant protection against various cancers including prostate cancer [6]. Quercetin, a flavonoid commonly present in many vegetables has been shown to induce apoptosis in many tumor cell lines [7]. It can interact with a broad range of enzymes specifically receptor kinases, protein kinase C, cyclin-dependent kinase's (Cdk's) and also with MEK-ERK signaling [8–10]. Previous studies from our laboratory demonstrated that quercetin inhibited the prostate cancer cell (PC-3) growth by down regulating pRb phosphorylation [11].

In this study we examined the effects of quercetin on protein levels of the MMP-2 and MMP-9 in *in vitro* using PC-3 cells. The PC-3 cell line is highly invasive and metastatic and androgen independent was derived from the ascites fluid of a patient with advanced prostate cancer that had metastasized to the lungs, pancreas, liver, kidney and bones [12]. This cell line possesses high invasive capacity by having more expressions of MMPs [13, 14]. Our study is aimed to investigate the role of quercetin on the levels of MMP-2 and MMP-9 in prostate cancer cells (PC-3) and this study suggesting its role as antimetastatic in cancer progression.

Methods

Minimum Essential Medium (MEM), Fetal Bovine Serum (FBS), trypan blue and quercetin were purchased from Sigma Chemical Co., USA. Thymidine [^{3}H] was purchased from BRIT, Mumbai, India. Other chemicals were obtained from Sisco Research Laboratories (SRL), India. All the chemicals used were extra pure and were culture grade. The androgen-independent prostatic carcinoma PC-3 cell line was obtained from National center for cell science, Pune, India.

Quercetin was dissolved in dimethyl sulfoxide (DMSO). DMSO in culture media never exceeded 0.1% (v/v), the concentration known not to affect the cell proliferation. Cell viability was tested by Trypan blue exclusion method. PC-3 cells were plated at 1×10^5 cells per well in 12-well plates in MEM containing 10% FBS. The growth inhibitory effect of quercetin was studied using thymidine [^{3}H] incorporation.

Cell proliferation

Cell proliferation was assessed by thymidine incorporation method [15]. DNA synthesis was assessed by incorporation of [^{3}H] thymidine in cell monolayers. During the final 4 h of quercetin treatment, 1 μCi/well [^{3}H] thymidine containing medium was added and incubated. Monolayers were rinsed twice with ice-cold saline and fixed with 1 ml/well ice-cold methanol-acetic acid mixture at 4°C for a minimum of 2 h. Cells were solubilized in 0.5 ml of sodium dodecyl sulfate (SDS) and 250 μl of each lysate was mixed with the scintillation fluid, before counting. The samples were counted using 1409 Wallac DSA liquid scintillation counter.

Preparation of conditioned medium (CM) and cell lysates (CL)

PC-3 cells exponentially growing in T-75 flasks (Nunc Costar) were collected by trypsin-EDTA treatment, washed with serum-containing medium and allowed to recover from trypsinization for at least 30 min at 37°C. Cells were then washed twice in serum-free MEM and diluted in the same medium supplemented with 0.1% Bovine serum albumin (BSA, fraction V, Sigma) to a density of 3×10^5 cells/ml and cultured for 24 h in the presence of the quercetin at concentrations of 50 and 100 μM. The resultant culture supernatants were collected and stored at −20°C. Cell monolayers were washed once with serum-free MEM and extracted by lysing the cell monolayer with SDS lysis buffer (2% SDS, 125 mM Tris-HCl, pH 6.8, and 20% glycerol).

Immunoblot analysis

The lysates were boiled for 5 min and then clarified by a 20-min centrifugation at 4 °C. Protein concentration was measured by the method of Lowry *et al*. [16]. Equal amount of protein samples in SDS sample buffer (1% SDS, 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 5%-mercaptoethanol, and 0.05% bromphenol blue) were boiled for 5 min and subjected to reducing 10% SDS-PAGE. After electrophoresis, the proteins were transferred to a PVDF membrane (Immobilon-P, Millipore, USA). The protein extract from conditioned media was subjected to 10% SDS-PAGE and equal loading was confirmed by Ponceau S stain (0.2% Ponceau S in 3% trichloro acetic acid). The membranes were blocked with phosphate buffered saline (PBS) containing 2% (w/v) BSA (fraction V, Sigma) and 0.1% (v/v) Tween 20 at room temperature for 60 min and then probed for 16 h at 4 °C with two distinct polyclonal rabbit antibodies directed against the human MMP-2 and MMP-9 (kindly provided by Dr. W.G. Stetler-Stevenson, NIH, MD). After three washes with PBS, the blot was incubated with the anti-rabbit-IgG horseradish peroxidase-conjugated secondary antibody. The antigen was detected using the western blot chemiluminescence Reagent

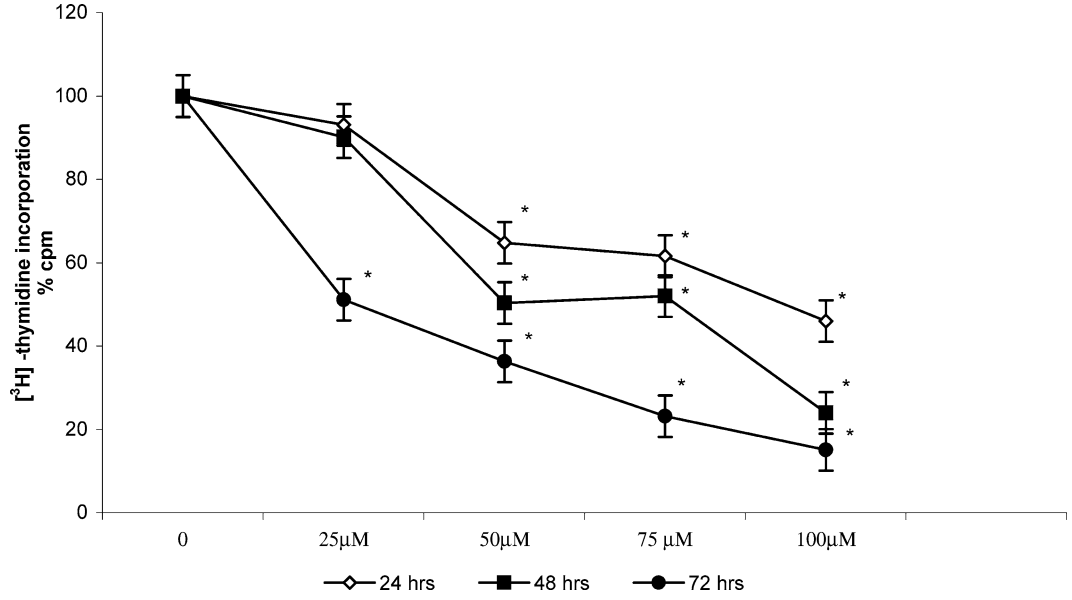

Fig. 1. Effect of quercetin on PC-3 cell proliferation. 3×10^3 cells were plated in 24-well plates. After reaching 70–80% confluence, in the presence of 5% FBS, cells were treated with vehicle or with various concentrations of quercetin (25, 50, 75, and 100 μM) for 24, 48 and 72 h. Proliferation of cells was quantitated by [^{3}H] thymidine incorporation. Experiments were performed in triplicate, values represents % of cpm and SEM was less than 10%. *represents statistical at $P < 0.05$ using SNK test between control and quercetin treatment groups.

Plus (PerkinElmer Life Sciences), according to the manufacturer's instruction.

Gelatin zymography

The cell lysates (75 μg) of control and quercetin treated cells were subjected to SDS-PAGE with 10% (w/v) acrylamide gel containing gelatin (0.6 mg/ml; Sigma Co, St Louis, USA). The gel was washed with washing buffer [50 mM Tris-HCl (pH 7.5), 0.15 M Sodium Chloride, 10 mM Calcium Chloride, 1 μM Zinc Chloride and 0.1% (v/v) Triton X-100] to remove SDS and then incubated at 37 °C in incubation buffer [50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 10 mM $CaCl_2$, and 1 μM $ZnCl_2$]. Thereafter, the gel was stained with Coomassie Brilliant Blue R-250, and gelatinolytic activity was detected as unstained bands on a blue background. The intensity of the bands were quantified with densitometry scanner (Bio Rad, USA).

Statistical analysis

The data were analyzed using the SPSS 7.5 Windows Students version software. For all the measurements, one-way ANOVA followed by Student's Newman Keuls test was used to assess the statistical significance of difference between control and quercetin-treated cells. A statistically significant difference was considered to be at $P < 0.05$.

Results

Cell proliferation

PC-3 cells showed a significant decrease in thymidine [^{3}H] uptake. Figure 1 shows the kinetics of proliferation upto 0–72 h quercetin treatments, during which the thymidine uptake was decreased between 3- and 4- fold in quercetin treated cells. Time response data demonstrate 50% growth inhibition at 100 μM for 24 h. Cell viability was also decreased in quercetin treated cells in a dose and time-dependent manner (Fig. 2).

Effects of quercetin on protein expression of MMP-2 and 9

Western blotting was performed to determine MMP-2 and MMP-9 proteins expression in quercetin treated PC-3 cells. The expression of active MMP-2 (72 kDa) and MMP-9 (83 kDa) proteins were significantly decreased in quercetin treated PC-3 cells in a dose-dependent manner (Fig. 3a). Western blot analysis also revealed that pro-MMP-9 (94 kDa) was significantly increased in 100 μM quercetin treated

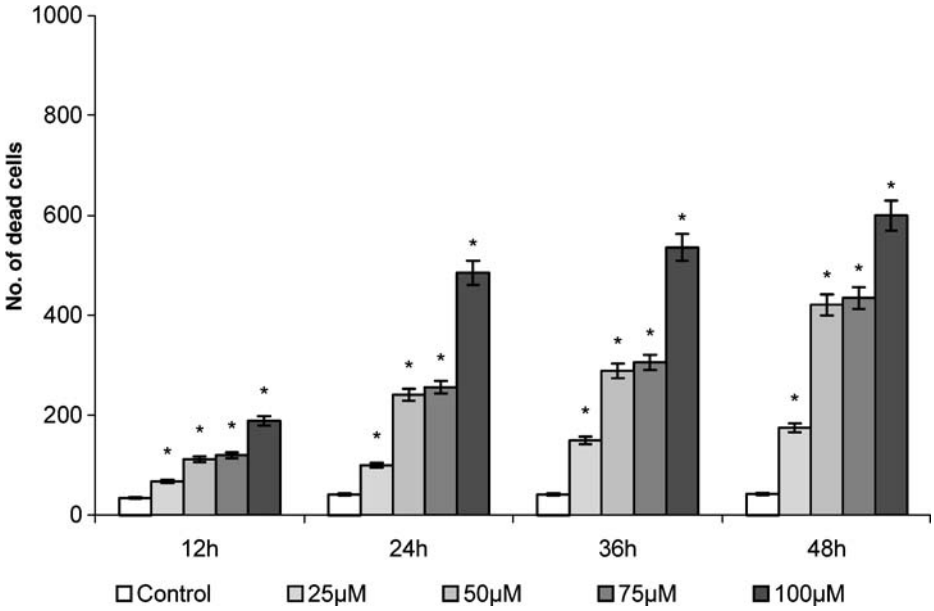

Fig. 2. Effect of quercetin on cell viability of PC-3 cells. The cell was performed by Trypan blue exclusion method. Values represents number of cells. Experiments were performed in triplicate, and SEM was less than 10%. *represents statistical at $P < 0.05$ using SNK test between control and quercetin treatment groups.

cells (Fig. 3a). Generally pro-MMP-9 is cleaved by other proteinases to yield a 83 kDa protein, which is an active enzyme.

Quercetin treated PC-3 cells at 50 and 100 μM for 24 h, also showed significant decrease in secretion of MMP-2 and 9 in conditioned media of PC-3 cells in a dose-dependent manner (Fig. 4a).

Gelatin zymography of MMP-2 and 9 activity in quercetin treated PC-3 cells

The effects of quercetin on MMP-2 and 9 enzyme activities were carried out by gelatin zymography. The results showed that quercetin decreased the activities of MMP-2 and 9 in PC-3 cells, whereas PC-3 cells exhibited the mild increase in the activity of pro-MMP-9 at 100 μM quercetin treatment (Fig. 5).

Discussion

MMPs have been implicated in processes leading to cancer invasion and metastasis [17–19] and may also play a major role in tumor angiogenesis [18]. These contentions are supported by the fact that the levels of some MMPs are elevated in several cancer types. These enzymes, therefore, appear to be the appropriate targets for the development of anti-cancer and anti-metastasis agents. MMP-2 and MMP-9 are powerful enzymes that are considered to be important contributors to the processes of invasive metastasis and angiogenesis in various tumors [19] including prostate cancer [20].

The bioflavonoid quercetin, which is widely distributed in fruits and vegetables, has been shown to be chemopreventive in cancer prevention in several animal models and cancer cell lines [21]. Flavonoids are competitive inhibitors for the ATP binding site on a variety of enzymes such as PKC [22], a region of considerable homology among kinases. Among the flavonoids tested, quercetin exerted the strongest inhibitory effects on cell growth, kinase activity and MMP secretion in many tumor cells [23]. Quercetin also inhibited PC-3 cells growth by down regulating pRB phosphorylation [11].

In the present investigation, a marked decrease in the secretion of the 72- and 83-kDa gelatinases from PC-3 cells in response to quercetin was observed. These two gelatinases appear to be type IV collagenases, i.e. MMP-2 and MMP-9, as demonstrated by western blotting analysis. Western blot analysis of the cell lysate obtained from 100 μM quercetin treated PC-3 cells revealed the presence of different immunoreactive MMP-9 species corresponding to a 94 kDa protein, previously identified as pro-MMP-9 [23]. In the presence of higher dose of quercetin, the capacity of PC-3 cells to process pro-MMP-9 was dramatically impaired, as judged by the increased levels of pro-MMP-9. Western blotting of conditioned media collected from the quercetin

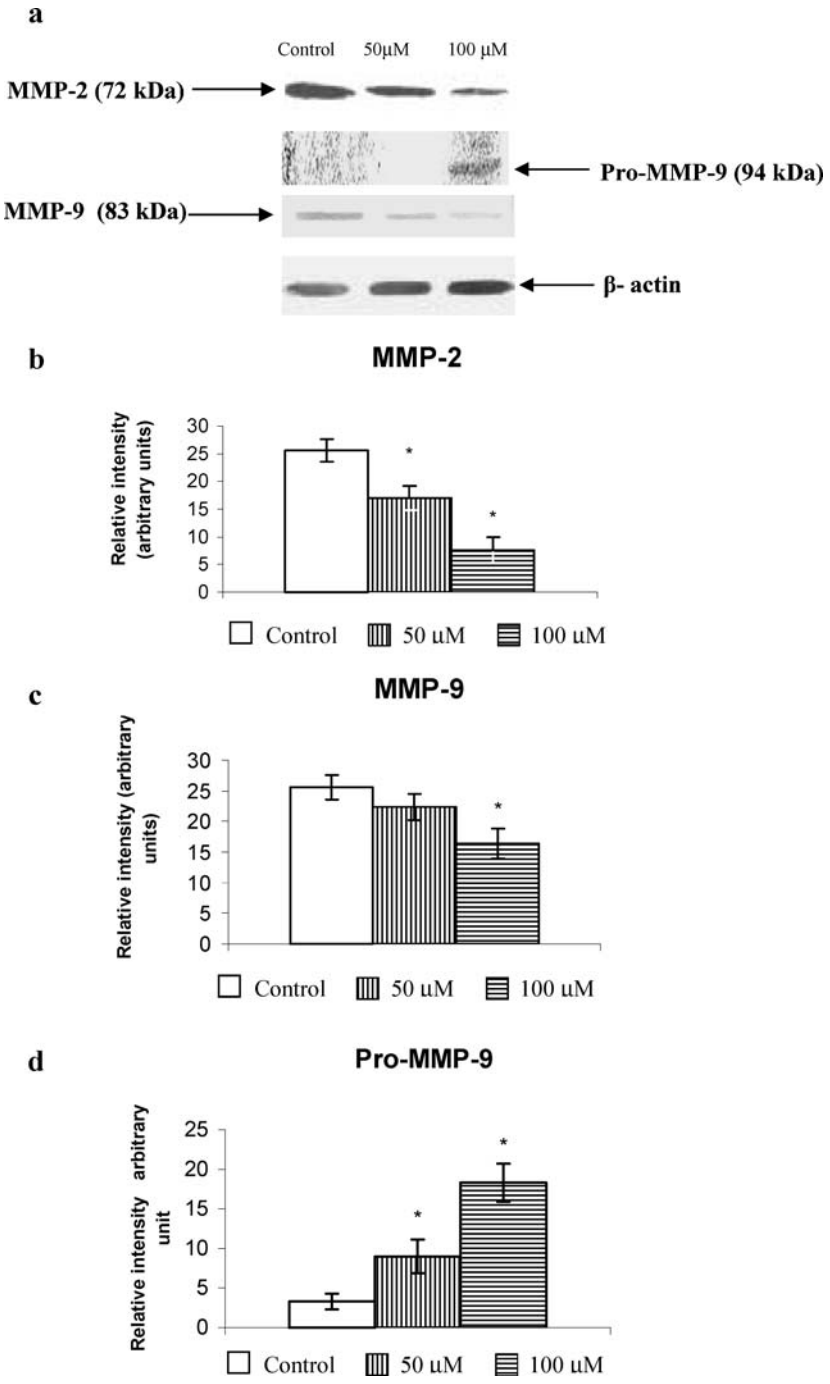

Fig. 3. (a) Effect of quercetin on MMP-2 and 9 proteins expression in cell lysate of PC-3 cells by immunoblot. PC-3 cells were treated with indicated concentrations of quercetin for 24 h. Cell lysates were analyzed by Western blotting. Blots were incubated with anti-MMP-2 and 9 Rabbit polyclonal antibodies and then incubated with anti Rabbit secondary antibody. Bands were developed with ECL kit (Perkin Elmer, USA). An anti-β-actin antibody was used to check the proper protein loading. Immunoreactive bands was analysed by densitometry, and the ratio of the expression of MMP-2, 9 and β-actin has been plotted. (b) shows the proteins levels of MMP-2 in quercetin treated cells. (c) shows the proteins levels of MMP-9 in quercetin treated cells. (d) shows the proteins levels of pro- MMP-9 in quercetin treated cells. Each value is mean $\pm$ SEM of three observations. * represents significance between control Vs quercetin treatment groups at $P < 0.05$ level using Student-Newman-Keuls test.

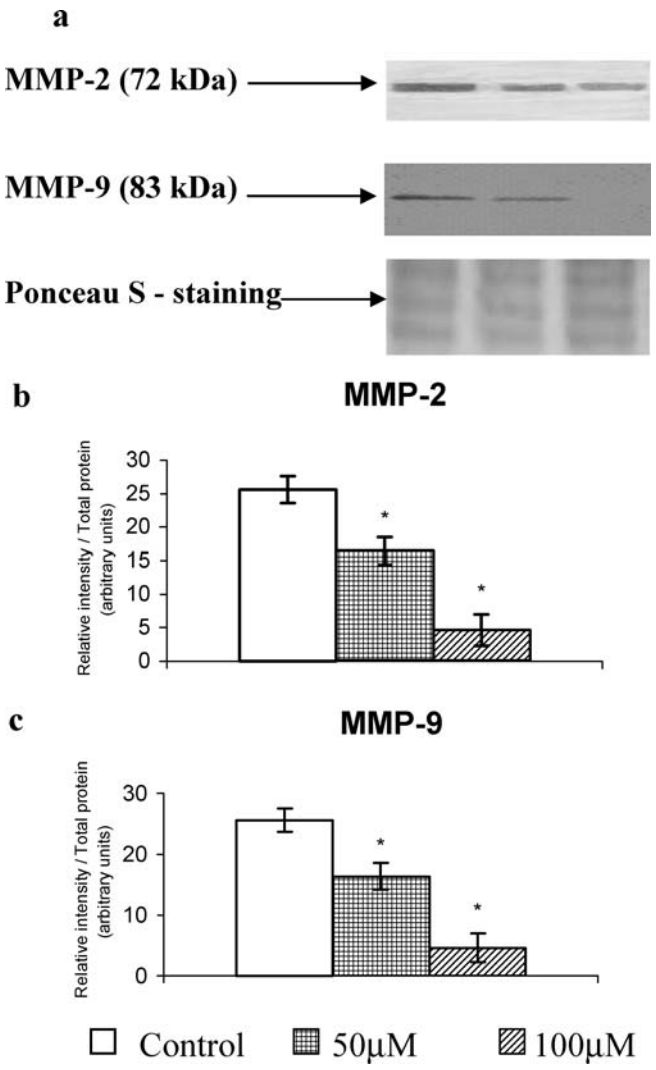

Fig. 4. Effect of quercetin on MMP-2 and 9 proteins expression in conditioned media of PC-3 cells by immunoblot. (a) PC-3 cells were treated with indicated concentrations of quercetin for 24 h conditioned media were analyzed by Western blotting. Blots were incubated with anti-MMP-2 and 9 Rabbit polyclonal antibodies and then incubated with anti Rabbit secondary antibody. Bands were developed with ECL kit (Perkin Elmer, USA). Ponceau S stain (0.2% Ponceau S in 3% Trichloro acetic acid) was used to check the proper protein loading. Immunoreactive bands was analysed by densitometry, and the ratio of the expression of MMP-2, 9 and total protein has been plotted. (b) shows the proteins levels of MMP-2 and (c) shows the protein levels of MMP-9 in quercetin treated cells. Each value is mean ± SEM of three observations. * represents significance between control Vs quercetin treatment groups at $P < 0.05$ level using Student-Newman-Keuls test.

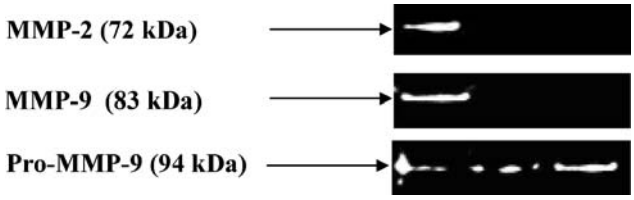

Fig. 5. The zymography of gelatinases was performed in cell lysates of control and quercetin treated cells as described in the experimental section. The figure is representative of three independent experiments carried out in duplicate, each of which demonstrated similar results.

treated PC-3 cells showed a significant decrease in the protein levels of MMP-2 and 9 in a dose-dependent manner. The zymography results also showed that decreased activities of MMP-2 and MMP-9 in quercetin treated PC-3 cells. It also revealed that cells treated with quercetin express mild increase in activity of pro-MMP-9, when compared with control. This indicates that quercetin inhibits conversion of pro-MMP-9 to MMP-9 in cultured human prostate cancer cells (PC-3). Quercetin may cause the inhibition of MMP-9 at activation or secretion.

MMP-9 and MMP-2 are expressed in various human epithelial cancers and their levels seem to be related to the metastatic potential and malignancy [24–28]. Synthetic peptides, which inhibit MMP activity can block malignant tumor growth in animals [29, 30]. The expression and activities of MMPs are regulated at several levels [31]. Studies over the past decade have shown that MMPs are also regulated by a variety of growth factors [22, 32, 33].

Insulin-like growth factors (IGFs) were found to induce the secretion of MMPs in tumor cells [34]. It was also demonstrated that IGF-I receptor regulated the secretion of MMP-9 and MMP-2 in tumor cells [35]. These results also raise the possibility that IGFs not only enhances the secretion of MMPs, but might also in turn enhance the metastatic potential of tumors. Conversely, agents that inhibit IGF-IR tyrosine kinase activity may play a potential role in the prevention of metastasis. Scholar and Toews [36] reported that the tyrosine kinase inhibitor genistein, a flavonoid could inhibit invasion by tumor cells, possibly via repression of tyrosine phosphorylation. Studies from our laboratory showed that quercetin decreased the levels of IGFs and increased the level of Insulin-like growth factor-binding protein-3 (IGFBP-3) [37].

The present study clearly showed that quercetin is a potent inhibitor of MMP-2 and 9 expressions. Previous studies have also shown that MMP-2 and 9 expressions were regulated by MAP kinase signaling pathways, including ERK and MAPK signaling cascades in human vascular endothelial cells [38]. It was reported that quercetin decreased the expression of MMP-9 via the protein kinase C pathway in murine melonoma cells [23]. It is well known that quercetin is an inhibitor of several kinases including MAP kinases and tyrosine kinases [39, 40]. Hence, it is reasonable to speculate that quercetin might have down regulated the expression of MMP-2 and -9 through inhibition of protein kinases. The cell lysate data also revealed that quercetin could retain the intracellular concentration of the pro-MMP-9. However, this

work could not rule out the possible mechanism behind the inhibition of activation of pro-MMP-9.

Taken together, the present study suggests that quercetin could inhibit secretion of MMP-2 and 9 from tumor cells and thereby reduce the potential for metastasis. The findings also suggest that this natural product may lead to growth inhibition of tumors and a reduced likelihood of cancer metastasis and also encourages the clinical investigation of quercetin in the prevention of cancer invasion. Targeting specific genes that regulate expression of MMPs could add advantage in the treatment of prostate cancer.

Acknowledgments

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