Quercetin Suppresses Intracellular ROS Formation, MMP Activation, and Cell Motility in Human Fibrosarcoma Cells

Dong Eun Lee, Min-Yu Chung, Tae Gyu Lim, Won Bum Huh, Hyong Joo Lee, and Ki Won Lee

Abstract: Cell metastasis is a major cause of death from cancer and can arise from excessive levels of oxidative stress. The objective of this study was to investigate whether the natural flavonoid quercetin can inhibit matrix metalloproteinase (MMP)-2 and -9 activities through the attenuation of reactive oxygen species (ROS) formation, an event expected to lead to the inhibition of cell motility. To induce sustained ROS formation, cells were treated with phenazine methosulfate (PMS; 1 μM). Noncytotoxic concentrations of quercetin inhibited PMS-induced increases in cell motility in HT1080 human fibrosarcoma (HT1080) cells. While nearly 100% of cells were observed to migrate after 24 h of PMS treatment, quercetin significantly ($P < 0.01$) suppressed this effect. We also found that quercetin, up to 10 μg/mL, attenuated PMS-induced MMP-2 activation. We then investigated whether the decreased levels of MMP-2 activation could be attributable to lower levels of ROS formation by quercetin. We found that quercetin treatments significantly attenuated PMS-induced ROS formation ($P < 0.01$) and resulted in decreased cell motility associated with a reduction in MMP-2 and -9 activitiy in HT1080 cells, even in the absence of PMS treatment. Collectively, these results suggest that quercetin inhibits cell motility via the inhibition of MMP activation in HT1080 cells in the presence and absence of PMS. This is likely to be a result of the suppression of intracellular ROS formation by quercetin.

Keywords: cell motility, MMP-2, quercetin, phenazine methosulfate, reactive oxygen species

Practical Application: Our findings demonstrate that quercetin has the potential to inhibit cancer cell metastasis by suppressing mechanisms relevant to cell motility. The protective role of quercetin is likely attributable to its anti-oxidant capacity.

Introduction

Metastasis is a critical event associated with an estimated 90% of deaths from human cancers. During cell migration, components of the basement membrane and extracellular matrix (ECM) are rearranged and degraded, leading to the ability of malignant cells to invade surrounding tissue and spread systemically (Coussens and others 2002). The rearrangement and degradation of the ECM can be initiated by various proteinases synthesized and secreted from tumor cells (Coussens and others 2002). Among the families of proteolytic enzymes, the activation of matrix metalloproteinase (MMP) has been implicated in a majority of human cancers (Egeblad and Werb 2002). Human MMPs are categorized by their substrate specificity and expression patterns (Radisky and Przybylo 2008). Of particular note are the gelatin-degrading MMPs, of which MMP-2 and MMP-9 are known to play a key role in cancer cell invasion and metastasis via the degradation of type IV collagen, a major component of the basement membrane (Ura and others 1989; Bjorklund and Koivunen 2005).

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Oxidative stress arises after sustained reactive oxygen species (ROS) production and is associated with cancer progression (Valko and others 2006). Excessive levels of hydrogen peroxide (H_2O_2) can result from increased manganese superoxide dismutase (MnSOD) activity through the activation of the redox-sensitive transcription factor NFκB, resulting in MMP activation (Essick and others 2011). However, subsequent oxidative stress-mediated MMP-2 and -9 activation can be ameliorated by antioxidants (Kelly and others 2008; Mateos and others 2008). The suppression of ROS and/or ROS-mediated oxidative damage can therefore play a role in preventing cancer metastasis.

Quercetin is a polyphenol found abundantly in a variety of foods including apples, onions, and green tea (Scalbert and Williamson 2000). Numerous health benefits of quercetin have been reported (Zhou and others 2005; Lee and others 2008a; Hwang and others 2009; Jang and others 2009), and the compound has been found to exhibit anticarcinogenic activity via multiple mechanisms (Jagtap and others 2009). As one of the most potent known ROS scavengers among the flavonoid family (Pocernich and others 2011), quercetin ameliorates ROS-mediated cellular damage (Jagtap and others 2009), and likely contributes to protection against various cancers. Recently, quercetin has also been shown to decrease MMP-2 and -9 expression (Vijayababu and others 2006; Hwang and others 2009), thereby preventing tumor invasion and metastasis. Guava leaf extract has been found to contain high levels of quercetin-3-glucoside and inhibits MMP-2 and -9, likely due to influences on the ERK1/2 MAPK signaling pathways (Im and

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others 2012). Given that MMP-2 and -9 can also be regulated by ROS-mediated oxidative stress (Jiang and others 2002; Kelly and others 2008; Mateos and others 2008; Yao and others 2009; Essick and others 2011), quercetin may play a role in suppressing MMP-2 and -9 activity leading to a preventive effect on cancer cell metastasis.

Phenazine methosulfate (PMS) is a superoxide anion-producing agent. In a previous study, the nitric oxide generator, peroxynitrite, and hydrogen peroxide (H_2O_2) when treated alone did not promote pro-MMP activation in HT1080 human fibrosarcoma (HT1080) cells (Yoon and others 2002). However, cells treated with nontoxic levels of PMS exerted increased pro-MMP-2 activation, cell motility, and tumor invasion (Yoon and others 2002). This suggests that PMS is an effective pro-MMP-2 activator that increases intracellular H_2O_2 production in HT1080 cells. In the present study, HT1080 cells were treated with PMS to investigate the role of quercetin as an intracellular ROS scavenger, and its influences on MMP-mediated cell motility. We also aimed to investigate whether quercetin inhibits cell motility in HT1080 cells as a possible result of spontaneous ROS overproduction and the attenuation of MMP activation.

Materials and Methods

Chemicals

Quercetin, PMS, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), dichlorofluorescein diacetate (DCF-DA), and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis. Mo., U.S.A.). Dulbecco's modified eagle medium (DMEM) and penicillin/streptomycin were obtained from GIBCO BRL (Grand Island, N.Y., U.S.A.).

Cell culture

HT1080 (human fibrosarcoma) cells were purchased from the Korean Cell Line Bank (Seoul, Korea). The cells were cultured in DMEM with 10% (v/v) FBS and penicillin/streptomycin at 37 °C in an atmosphere of 5% CO_2.

MTT assay

Quercetin cell cytotoxicity was measured using an MTT assay. HT1080 cells were cultured until confluence in 96-well plates, and different concentrations of quercetin (0, 5, and 10 μg/mL) were treated in the presence or absence of PMS (37 °C, 48 h). MTT solution (20 μL) was added and the cells were incubated for 4 h. The media was removed and 200 μL of DMSO was added to dissolve the formazan residue. After shaking for 15 min, the optical density (OD) was estimated at 570 nm using a microplate reader (Emax, Molecular Devices, Sunnyvale, Calif., U.S.A.).

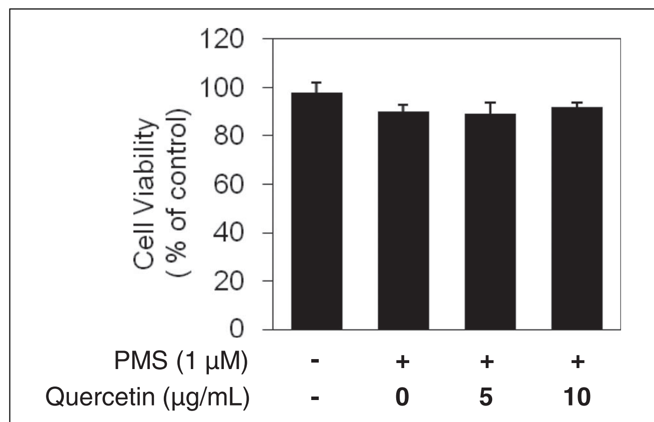

Figure 1–Effect of PMS and quercetin on cell viability in HT1080 cells. Treatment with PMS at 1 μM concentration and quercetin up to 10 μg/mL did not result in observable cytotoxicity in HT1080 cells. Results are expressed as cell viability relative to the untreated controls, as determined from 3 independent experiments. Values are expressed as mean ± SD.

Gelatin zymography

The expression levels of MMP-2 and -9 were evaluated using gelatin zymography as previously described (Lee and others 2008b). In brief, HT1080 cells were cultured until 90% confluence, and starved with serum-free DMEM for 24 h. Cells were treated with varying concentrations of quercetin (0, 5, and 10 μg/mL) for 48 h in the presence and absence of PMS (1 μM), and the culture media was collected. Protein lysate from the culture was measured using BCA protein assay reagents (Pierce, Rochford, Ill., U.S.A.). The samples were then mixed with loading buffer [10% SDS, 25% glycerol, 0.25 M Tris (pH 6.8), and 0.1% bromophenol blue], and separated on a 10% SDS-PAGE gel. The gel was washed with renaturing buffer (Invitrogen, Carlsbad,

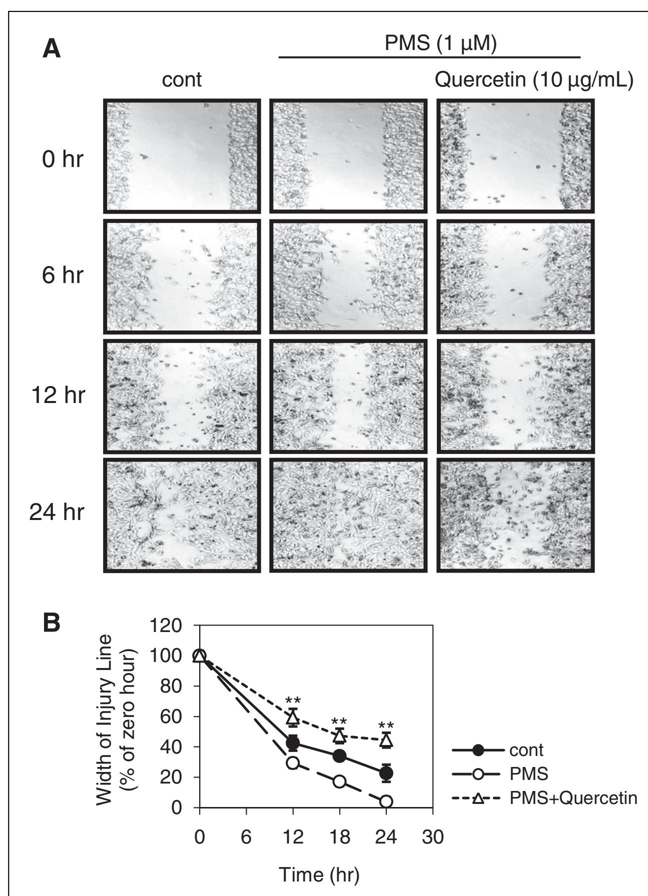

Figure 2–Effect of quercetin on PMS-induced cell motility in HT1080 cells. (A) Quercetin inhibited restoration of wound healing after 24 h of treatment. The migratory capacity of the cells was evaluated using a wound healing assay, in the presence or absence of quercetin in PMS-treated cells. (B) Quantitation of the assay data reveals that quercetin attenuates the width of injury line to a lesser extent than that observed in untreated controls. The width of the injury line was measured across 3 independent experiments, and plotted as a percentage of the width at 0 h. Values are expressed as mean ± SD. The asterisk (**) indicates a significant difference ($P < 0.01$) compared to the group treated with PMS only.

Calif., U.S.A.) for 30 min (25 °C) and incubated with developing buffer (Invitrogen) for 24 h (37 °C). The areas of gelatinase activity were visualized by staining the gels with 0.5% Coomassie brilliant blue.

Wound migration assay

Wound migration assays were performed as previously described (Lee and others 2008b). Briefly, HT1080 cells were pretreated with mitomycin C (25 μg/mL) for 30 min to suppress cell proliferation, plated on culture dishes, and grown until 90% confluence. The single cell layer was then damaged by making an injury line using a tip of 2 mm width. The plates were rinsed with PBS followed by the addition of complete media. The cells were then allowed to migrate to fill the gap for 24 h.

Three images were randomly captured at the indicated time-points from each well using an inverted microscope at the indicated time points (×100 magnification; Olympus Ix70, Okaya, Japan). To quantify the width of the injury line, 10 straight-distances of injury were randomly chosen and measured in each image using Image J software (NIH). The average values for 3 images from independent experiments were used for statistical analysis.

DCF-DA assay

To assess the effect of quercetin on intracellular ROS generation, DCF-DA assays were performed. Cells grown as monolayers were preincubated with PBS supplemented with 50 μM DCF-DA, and incubated at 37 °C for 30 min. After the supernatant was removed, fresh PBS supplemented with varying concentrations of quercetin was added, and incubated at 37 °C for a further 15 min. Relative fluorescence was measured over time (0.5 to 20 min) using a fluorescence spectrophotometer F-4500 (Hitachi, Ltd., Tokyo, Japan) operating at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

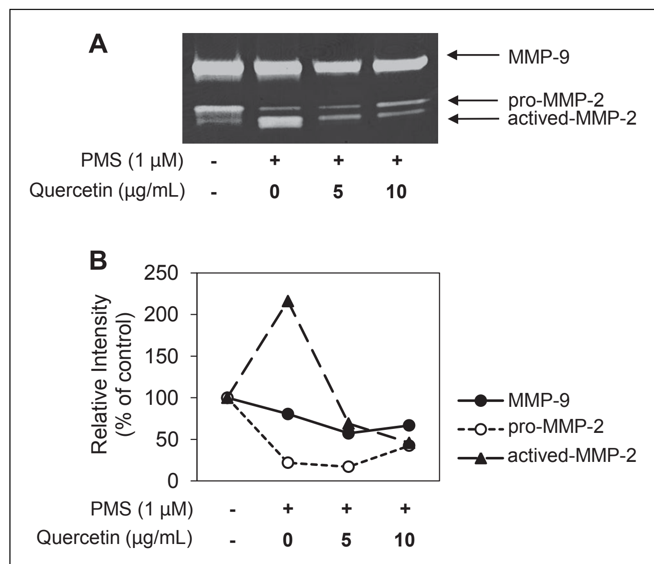

Figure 3–Effect of quercetin on MMP-2 and -9 activation in HT1080 cells treated with PMS. (A) Treatment of PMS at 1 μM increased MMP-2 activation, which was then attenuated in the presence of quercetin (10 μg/mL). MMP-9 activation remained unaffected by PMS or quercetin treatment. (B) Data quantitation reveals that quercetin suppresses PMS-induced activation of pro-MMP-2. The relative enzyme activities of MMP-9, pro-MMP-2, and activated-MMP-2 are expressed as a percentage relative to the untreated controls.

Statistical analysis

Data are expressed as mean ± SD. Student's *t*-test was used for comparisons between groups. A probability value of $P < 0.05$ and $P < 0.01$ were used as the criterion for statistical significance.

Results and Discussion

Quercetin does not affect cell viability in HT1080 cells

Cell cytotoxicity after treatment with PMS and quercetin was measured using MTT assay. We found that treatments of PMS at 1 μM and quercetin at 5 and 10 μg/mL did not inhibit viability of the HT1080 cells (Figure 1). Thereafter, PMS at 1 μM and quercetin up to 10 μg/mL concentrations were used to evaluate ROS-mediated changes in MMP expression and cell migration in HT1080 cells.

Quercetin suppresses PMS-dependent increases in cell motility in HT1080 cells

We next examined the effect of quercetin on PMS-induced cell motility using an *in vitro* wound migration assay as previously

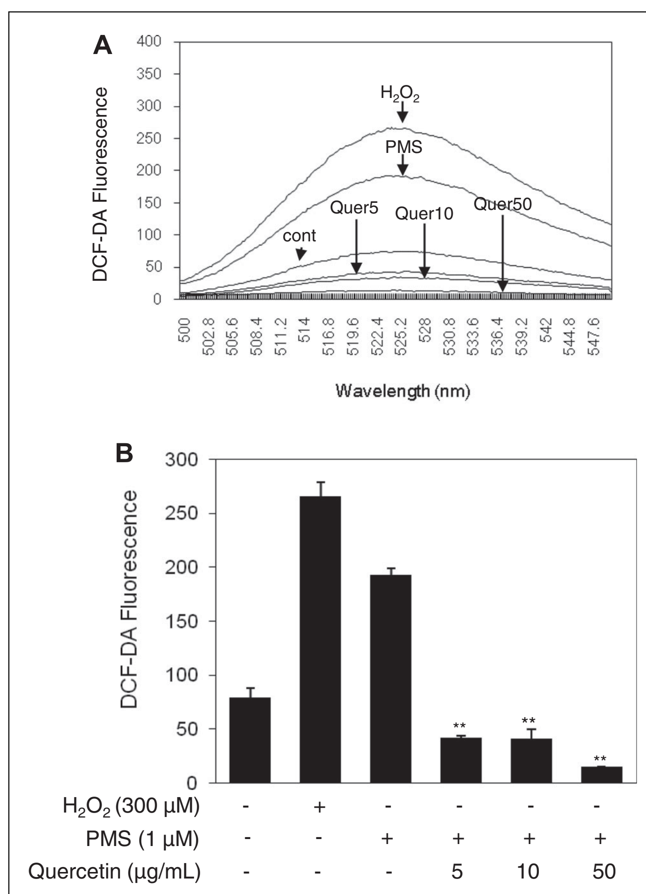

Figure 4–Effect of quercetin on intracellular ROS formation following PMS treatment. (A) Quercetin abrogates PMS-induced intracellular ROS production. Intracellular ROS levels were determined by DCF-DA assay. Cells were treated with 5, 10, and 50 μg/mL of quercetin in the presence or absence of 1 μM PMS. Cells treated with H_2O_2 (300 μM) were used as a positive control. (B) Quantitation of the data shows that quercetin attenuates intracellular ROS formation in PMS-treated HT1080 cells. The intracellular ROS contents of each group were measured over 3 independent experiments, quantified and plotted. Values are expressed as mean ± SD ($n = 3$ per group). The asterisk (**) indicates a significant difference ($P < 0.01$) compared to the cells treated with PMS only.

described (Soderholm and Heald 2005). Compared to control cells, PMS-treated HT1080 cells exhibited markedly increased cell motility after 24 h from the time of wound scratching, an effect that was decreased in the presence of co-treatment with a nontoxic concentration of quercetin (10 μg/mL) (Figure 2A).

The width of the injury lines was quantified and found to be almost 100% recovered in the cells treated with PMS (Figure 2B). In contrast, treatment of quercetin (10 μg/mL) in PMS-treated HT1080 cells resulted in a decrease of injury line recovery. The width of these injury lines was decreased by 50%, indicating that half of the cells treated with quercetin spread onto the injury line after 24 h of quercetin treatment (Figure 2A). The reduction was statistically significant compared to the groups treated with PMS only ($P < 0.01$). These results indicate that quercetin attenuates cell motility induced by PMS in HT1080 cells.

Quercetin attenuates increases in expression levels of activated-MMP-2 caused by PMS treatment in HT1080 cells

We then aimed to determine whether quercetin-mediated decreases in cell motility were associated with decreased MMP activity, as ROS-triggered oxidative stress is known to increase MMP activity (Jiang and others 2002; Kelly and others 2008; Mateos and others 2008; Yao and others 2009; Essick and others 2011). To measure MMP activation, gelatin zymography was performed. Consistent with previous studies (Yoon and others 2002), PMS (1 μM) induced the activation of pro-MMP-2, which was evidenced by greater expression of activated-MMP-2 and lower expression levels of pro-MMP-2 (Figure 3A and B). Meanwhile, cells treated without PMS showed the opposite trend to that observed in the PMS-treated HT1080 cells (Figure 3A and B). This indicates that PMS increases catalytic activity of MMP-2, but not MMP-9. This finding is in agreement with a previous study reporting that PMS activates MMP-2, but not MMP-9 (Yoon and others 2002). Quercetin at 5 and 10 μg/mL markedly suppressed the expression levels of activated MMP-2, induced by PMS. However, MMP-9 was largely unaffected by quercetin treatment (Figure 3B). Collectively, these results suggest that quercetin inhibits PMS-induced cell migration by attenuating MMP-2 activation, which would otherwise be induced by PMS treatment in HT1080 cells.

Quercetin suppresses PMS-induced intracellular ROS formation in HT1080 cells

We then examined whether decreased MMP-2 activity and subsequent suppression of cell motility was associated with the ROS-scavenging capacity of quercetin. Intracellular ROS formation was evaluated using a DCF-DA assay. We observed that quercetin treatment inhibited PMS-induced increases in intracellular ROS formation in HT1080 cells (Figure 4A). Quantification of the data indicated that quercetin at 5, 10, and 50 μg/mL significantly

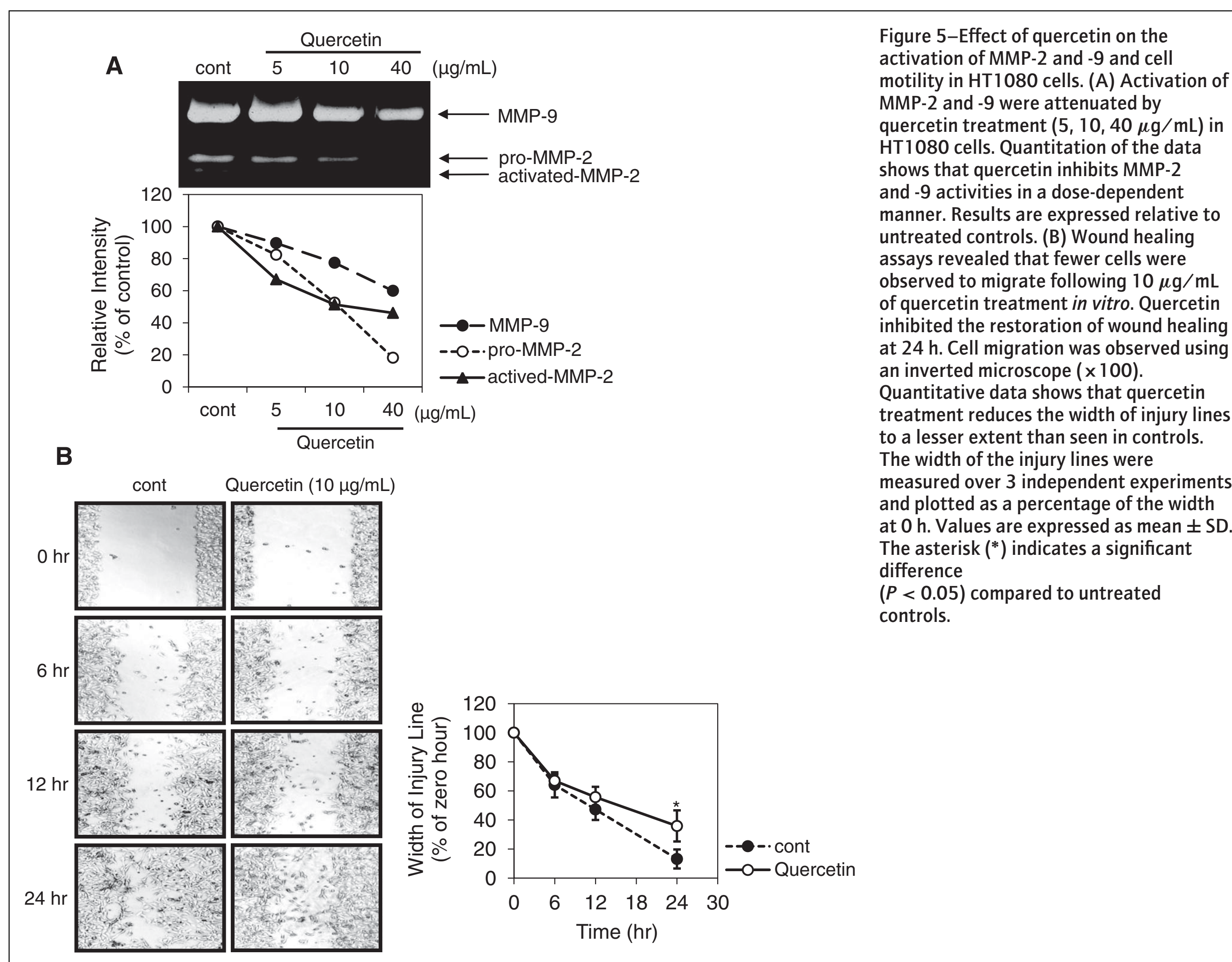

Figure 5–Effect of quercetin on the activation of MMP-2 and -9 and cell motility in HT1080 cells. (A) Activation of MMP-2 and -9 were attenuated by quercetin treatment (5, 10, 40 μg/mL) in HT1080 cells. Quantitation of the data shows that quercetin inhibits MMP-2 and -9 activities in a dose-dependent manner. Results are expressed relative to untreated controls. (B) Wound healing assays revealed that fewer cells were observed to migrate following 10 μg/mL of quercetin treatment *in vitro*. Quercetin inhibited the restoration of wound healing at 24 h. Cell migration was observed using an inverted microscope (×100). Quantitative data shows that quercetin treatment reduces the width of injury lines to a lesser extent than seen in controls. The width of the injury lines were measured over 3 independent experiments and plotted as a percentage of the width at 0 h. Values are expressed as mean ± SD. The asterisk (*) indicates a significant difference ($P < 0.05$) compared to untreated controls.

attenuated PMS-induced ROS formation by 78.2%, 79.0%, and 92.4%, respectively, and these reductions were statistically significant ($P < 0.01$; Figure 4B).

We could not rule out the possibility that quercetin could be acting as a small molecule inhibitor on intracellular signaling pathways, in addition to its actions as a ROS scavenger. It has been demonstrated that PMS-induced pro-MMP-2 activation is regulated by intracellular signaling pathways such as the phosphatidylinositol 3-kinase (PI3K) and NFκB-mediated pathways following initial induction via membrane type 1-MMP (MT1-MMP) and TIMP2 (Yoon and others 2002). Indeed, quercetin has been reported to attenuate TNF-α-induced MMP-9 activation via direct inhibition of PI3K (Hwang and others 2009). We found that quercetin (10 and 20 μg/mL) mitigates PMS-induced Akt phosphorylation in HT1080 cells (Supporting Information Figure 2A). Therefore, quercetin-mediated inhibition of the Akt/PI3K signaling cascade may contribute to the deactivation of MMP-2 and/or MMP-9, and subsequent cell motility. Quercetin has also been reported to inhibit MMP-9 and subsequent tumor invasion by suppressing activation of the PKC delta/ERK/AP-1-signaling cascade by TPA treatment in MCF-7, a breast cancer cell line (Lin and others 2008). We found that quercetin (10 and 20 μg/mL) reduces PMS-mediated phosphorylation of JNK, but not ERK in HT1080 cells (Supporting Information Figure 2B). In addition, quercetin has been shown to influence NFκB translocation, thereby inhibiting MMP-2 activation and cell invasion in glioma cells (Park and Min 2011). Further studies are warranted to investigate quercetin's potential for regulating signaling pathways to suppress MMP-2 and -9, and thereby suppress subsequent cell motility independent from its effects on intracellular ROS formation.

Quercetin inhibits cell motility and suppresses MMP-2 and -9 activation in HT1080 cells in the absence of PMS

We next sought to investigate whether quercetin inhibits cell motility in HT1080 cells in association with spontaneous ROS overproduction and the attenuation of MMP activation. Compared to controls, noncytotoxic concentrations of quercetin (5, 10, 40 μg/mL) resulted in greater suppression of MMP-9, and pro- and activated-MMP-2 activity in HT1080 cells (Figure 5A). Quantitation of the data confirmed that the expression levels of activated-MMP-2 and MMP-9 were decreased by quercetin in a dose-dependent manner (Figure 5A). Of particular note, the expression levels of pro-MMP-2 was markedly attenuated by the treatment of quercetin (5, 10, and 40 μg/mL by 20%, 40%, and 80%, respectively) (Figure 5A).

Activation of MMP-2 and -9 has been reported to lead to the rearrangement and degradation of the ECM, leading to cancer cell migration (Bjorklund and Koivunen 2005; Pasco and others 2005; Itoh 2006). We measured the effect of quercetin on cell motility, and found that the quercetin-mediated decrease in MMP-2 activation was associated with a decrease in cell motility in HT100 cells (Figure 5B). It is likely that the cells treated with 10 μg/mL of quercetin exhibited less restorative capacity after injury, compared to control cells (Figure 5B). Data quantitation showed that HT1080 cells treated with quercetin for 12 h tended to migrate into the injury line to a lesser extent than control cells treated with no quercetin (Figure 5B). The difference in the width of the injury line between the quercetin-treated group and controls was statistically significant at 24 h after treatment ($P < 0.05$). Taken together, these results suggest that quercetin suppresses the activity of MMP-2 and -9, two major proteolytic enzymes during cell metastasis (Bjorklund and Koivunen 2005), which likely contributes to the inhibition of cell motility in HT1080 cells.

The HT1080 cell line is tumorigenic, and frequently used to investigate the inhibitory effects of anticancer agents on cell motility and invasion (Sun and others 2012). In fact, HT1080 cells even in the absence of treatment have elevated ROS production, which increases PKC and ERK phosphorylation, and subsequent MMP-2 activation and cell invasion (Sun and others 2012). We believe that HT1080 cells without PMS treatment are a more accurate representation of the physiology of cancer in patients. Therefore, we examined the effects of quercetin on HT1080 cells without PMS treatment, and we also observed the inhibitory effect of quercetin on cell motility. This inhibitory effect is likely attributable to decreased MMP-2 and MMP-9 activation, possibly as a result of the ROS-scavenging effects of quercetin.

Conclusion

Cell metastasis is a major factor that leads to cancer-related deaths, and its inhibition may contribute to more effective therapeutic strategies. In the current study, we investigated the role of the antioxidant quercetin on motility of HT1080 cells and found that cell motility induced by PMS was inhibited in association with MMP-2 activation. This effect was also associated with decreased intracellular ROS levels. In addition, quercetin inhibited MMP-2 and -9 activation and suppressed cell motility even without sustained H_2O_2 induction.

Despite multiple lines of evidence for quercetin's inhibitory effects against cancer and other chronic diseases, evidence from clinical studies remains limited. Given that 90% of cancer deaths are attributable to tumor invasion and metastasis, further research is justified in order to elucidate the anti-invasive and anti-metastatic activities of quercetin.

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Conflict of Interest

None of the authors declare any conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Supplementary Figure 1. Effect of PMS and quercetin on the cytotoxicity of HT1080 cells.

Supplement Figure 2. Effects of quercetin on the phosphorylation of Akt, JNK, and ERK in HT1080 cells.