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ORIGINAL ARTICLE

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Quercetin inhibits the invasion of murine melanoma B16-BL6 cells by decreasing pro-MMP-9 via the PKC pathway

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Abstract *Purpose*: On the basis of the inhibitory effect of quercetin on the invasion of melanoma B16-BL6 cells previously reported by us, the mechanisms of quercetin-mediated inhibition of invasion were further investigated in the present study. *Methods*: The ability of B16-BL6 cells to invade and migrate was evaluated in terms of the numbers of cells penetrating a reconstituted basement membrane in the Transwell coculture system. The relative levels and activities of matrix metalloproteinase-9 (MMP-9) and MMP-2 were determined by gelatin zymography and quantified using LabWorks 4.0 software. *Results*: The quercetin-mediated inhibition of invasion was partially blocked by phorbol-12,13-dibutyrate (PDB), a PKC (protein kinase C) activator, and by doxorubicin, a PKC inhibitor. Only the proforms of MMP-9 (92 kDa) and MMP-2 (72 kDa) were detected by gelatin zymography. Quercetin dose-dependently decreased the gelatinolytic activity of pro-MMP-9. Doxorubicin also markedly reversed the quercetin-induced decrease. Quercetin showed a dose-dependent antagonism of increases in gelatinolytic activity of pro-MMP-9 induced by PDB and free fatty acid (another PKC activator). *Conclusions*: Together with the report that quercetin directly reduces PKC activity, the results reported here suggest that quercetin may inhibit the invasion of B16-BL6 cells by decreasing pro-MMP-9 via the PKC pathway.

Keywords Quercetin · Melanoma B16-BL6 cells · MMP · PKC · Invasion

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Introduction

Metastasis, one of the major reasons for cancer mortality, is a multistep process including adhesion, invasion and migration. During the process, the metastatic tumor cells encounter various host cells, extracellular matrix and basement membrane components [1]. This encounter may be regulated by many factors, such as adhesion molecules, proteinases, and the cytoskeleton [2]. Some of the critical molecules in the process may be potential targets for evaluating antimetastatic agents. For example, matrix metalloproteinases (MMPs), secreted by tumor cells or other paracrine cells, play a critical role in the invasion of tumor cells [3, 4]. These enzymes are capable of degrading the extracellular matrix and basement membrane so that tumor cells can penetrate the physical barrier of blood vessels. In addition, MMPs also facilitate angiogenesis and proliferation of tumor [5, 6]. Pro-MMP-9 is a marker of liver metastasis in colorectal cancer and survival in advanced ovarian cancer [7, 8].

The synthetic low molecular weight MMP inhibitors, batimastat (BB-94) and marimastat (BB-2516), have a collagen-mimicking hydroxamate which facilitates chelation of a zinc ion into the active site of MMP [9]. BB-94 in a phase I trial has displayed a good effect against malignant pleural effusions, while BB-256 in a randomized double-blind placebo-controlled trial significantly improved survival of patients with inoperable colorectal hepatic metastases, and in a phase II trial also displayed activity in patients with metastatic malignant melanoma [10, 11, 12]. Arg-Gly-Asp-Ser (RGDS), a sequence in the central cell-binding domain of fibronectin, has also been shown to block the attachment between adhesion molecules and their receptors. These findings suggest that affecting metastasis-related enzymes, adhesion molecules or extracellular matrix may contribute to the control of metastasis.

The expression and activity of enzymes and adhesion molecules are regulated by kinases such as protein

kinase C (PKC) which is known to be involved in the transduction of signals for cell proliferation and differentiation, and plays an important role in carcinogenesis, tumor development and metastasis [13, 14, 15]. The activity of PKC is modulated by many small compounds including activators, such as phorbol-12,13-dibutyrate (PDB) and free fatty acid (FFA), and inhibitors, such as doxorubicin and chlorpromazine, in various ways. PDB can substitute for diacylglycerol (DAG) to greatly increase the affinity of the enzyme for Ca^{2+} as well as phospholipid, while FFA can increase the activity by regulating Ca^{2+} efflux and Ca^{2+} channels [16, 17, 18]. In contrast, doxorubicin inhibits the activity by interaction with DAG, and chlorpromazine by reducing the basal levels of cytoplasmic free Ca^{2+} [19, 20, 21]. Bryostatins, naturally occurring macrocyclic lactones, have also been shown in a phase II clinical trial to reduce metastasis of renal cell carcinoma by regulating the activity of PKC [22, 23].

Furthermore, some herbal drugs and the chemical compounds derived from natural sources have also been shown to be able to modulate the metastasis process [24, 25]. For example, quercetin, a bioflavonoid widely distributed in a variety of plants, has been shown to have anticancer and antioxidative activities [26, 27]. To confirm the effect of quercetin on tumor metastasis, we investigated its effect on the invasion and mobility of murine melanoma B16-BL6, a highly metastatic cell line, and found that it produced significant inhibition by arresting the cell cycle and inducing apoptosis by decreasing Bcl-2 expression [28]. The present study was, therefore, designed to further elucidate the relationship between the anti-invasive activity of quercetin and PKC.

Materials and methods

Cells and reagents

The highly metastatic cell line, murine melanoma B16-BL6, was obtained by in vitro selection for invasion. This cell line was maintained as monolayer cultures in RPMI-1640 (Gibco, BRL) medium containing 2 m*M*L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and supplemented with 10% fetal calf serum (FCS). The following reagents were purchased: fibronectin, Matrigel, PDB, bovine serum albumin (BSA), proteinase K and Coomassie brilliant blue R250, doxorubicin (Sigma); and FFA and chlorpromazine (Shanghai Chemical Reagent Company, Shanghai, China).

Invasion assay

The invasive activity of the tumor cells was assayed in a Transwell cell culture chamber (Costar 3422; Costar, Cambridge, Mass.). Polyvinylpyrrolidone-free polycarbonate filters with a pore size of 8.0 μm (Nucleopore, Pleasanton, Calif.) were coated with 2.5 μg fibronectin in a volume of 50 μl on the lower surface and dried at room temperature. Reconstituted basement membrane Matrigel was applied to the upper surface of the filter (5 μg/filter) to form a matrix barrier. Coated filters were washed with phosphate-buffered saline and dried immediately before use. Tumor cells in exponential growth were harvested with RPMI-1640 medium supplemented with 0.1% BSA. Tumor cell suspensions (1×10^6 cells/ml, 100 μl) were added to the upper compartment of the chamber and incubated for 10 h. After incubation, the filters were harvested, fixed with methanol and stained with hematoxylin and eosin. Tumor cells on the upper surface of the filters were removed by wiping with cotton swabs. Cells which had invaded through the Matrigel and the filter to various areas of the lower surface were counted under a microscope in five predetermined fields at a magnification of ×200, and were labeled at random with a marker pen before being counted. Each assay was performed in triplicate, and repeated at least three times.

Migration assay

Tumor cell migration along a gradient of substratum-bound fibronectin was assayed in a Transwell cell culture chamber according to a previously reported method [29]. The filters with a pore size of 8.0 μm were precoated with 5 μg fibronectin in a volume of 50 μl on the lower surface as described above. The cells (1×10^5 cells in 100 μl RPMI-1640 medium) were added to the upper compartment of the Transwell cell chamber and incubated at 37°C for 4 h. The subsequent procedures were the same as those for the invasion assay. Each assay was performed in triplicate, and repeated at least three times.

Gelatin zymography assay

B16-BL6 cells (1×10^7/ml) in exponential growth were suspended in RPMI-1640 medium containing 10% FCS and incubated in the presence of quercetin without or with PDB, FFA, doxorubicin or chlorpromazine at 37°C for 10 h. The supernatants (10 μl) were removed from the total culture medium (200 μl) and mixed with 5 μl sample buffer (62.5 m*M* Tris containing 10% glycerol, 0.00125% bromophenol blue, 12% SDS) without reducing reagent, and subjected to SDS-PAGE at 4°C for 1 h in 5% polyacrylamide gels that were copolymerized with 1 mg/ml of gelatin. After electrophoresis, the gels were washed twice in rinsing buffer (2.5% Triton X-100, 1 m*M* $CaCl_2$, 1 μ*M* $ZnCl_2$, 0.05% NaN_3) for 1 h at room temperature to remove SDS and were then incubated for 36 h at 37°C in 50 m*M* Tris buffer containing 5 m*M* $CaCl_2$, 1 μ*M* $ZnCl_2$ and 0.05% NaN_3. The gels were stained with 0.25% Coomassie brilliant blue R250 for 30 min, and destained for 8 h in 10% acetic acid and 30% methanol. The proteolytic activity was visualized as clear bands (zones of gelatin degradation) against the blue background of stained gelatin.

Statistical analysis

One-way analysis of variance (ANOVA) for multiple comparisons was used to determine whether there were any significant differences between the different treatments. Once significant differences had been detected ($P < 0.05$), Student two-tailed *t*-test was used to evaluate differences between two groups. All experimental results are shown as means ± SD.

Results

Effect of PKC activators and inhibitors on quercetin-induced inhibition of B16-BL6 cell invasion and mobility

B16-BL6 cells were incubated with or without quercetin (3.3×10^{-1} m*M*) in the presence or absence of PDB (100 n*M*), FFA (6.7 m*M*), doxorubicin (8.6 μ*M*) or chlorpromazine (14 m*M*) for 10 h at 37°C. As shown in Fig. 1, quercetin significantly inhibited the ability of the B16-BL6 cells to penetrate the reconstituted basement membrane. PDB significantly antagonized the

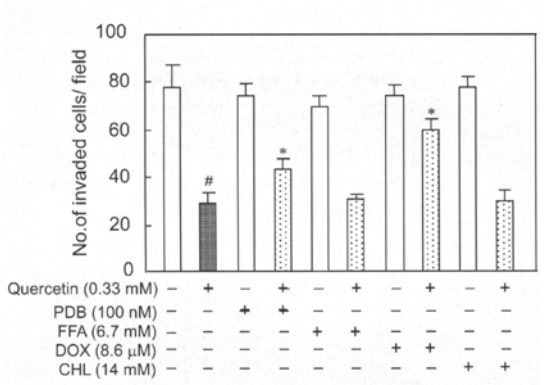

Fig. 1 Effect of PKC activators and inhibitors on quercetin-induced inhibition of B16-BL6 cell invasion. Polyvinylpyrrolidone-free polycarbonate filters with a pore size of 8.0 μm were coated with fibronectin on the lower surface. Reconstituted basement membrane Matrigel was applied to the upper surface of the filter to form a matrix barrier. B16-BL6 cells were incubated with quercetin (3.3×10^{-1} m*M*) in the presence or absence of PDB (100 n*M*), FFA (6.7 m*M*), doxorubicin (8.6 μ*M*) or chlorpromazine (14 m*M*) in the upper compartment of a Transwell culture chamber for 10 h. The invasive cells were determined as described in Methods. The results are expressed as the means ± SD of three independent experiments and each experiment was performed in triplicate. One-way ANOVA revealed a significant effect at $P<0.01$. $^{\#}P<0.01$, vs normal; $^{*}P<0.05$, vs quercetin alone (Student's two-tailed *t*-test)

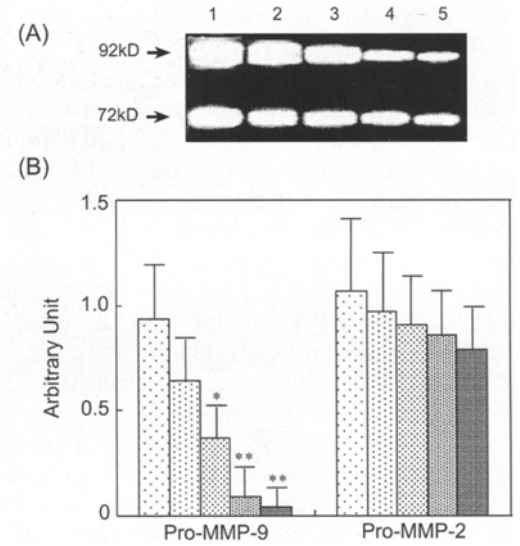

Fig. 2A, B Effects of quercetin on the gelatinolytic activities of MMP-9 and MMP-2 as determined by the gelatin zymography assay. **A** B16-BL6 cells were incubated with various concentrations of quercetin at 37°C for 10 h. Supernatants were collected and subjected to SDS-PAGE. After electrophoresis, the gels were washed, incubated, stained and destained as described in Methods. The results are representative of three independent experiments. *Lane 1* control; *lanes 2–5* 3.3×10^{-4}, 3.3×10^{-3}, 3.3×10^{-2}, and 3.3×10^{-1} m*M* quercetin, respectively. **B** The relative gelatinolytic activities of pro-MMP-9 and pro-MMP-2 were quantified densitometrically using LabWorks 4.0 software in relation to the activity without quercetin (control band assigned a value of 1). The results are expressed as the means ± SD of three experiments. One-way ANOVA revealed a significant effect at $P<0.01$. $^{*}P<0.05$, $^{**}P<0.01$, vs control (Student's two-tailed *t*-test). *Five bars from left to right, respectively: (1)* control, *(2)* quercetin 3.3×10^{-4} m*M*, *(3)* quercetin 3.3×10^{-3} m*M*, *(4)* quercetin 3.3×10^{-2} m*M*, *(5)* quercetin 3.3×10^{-1} m*M*

quercetin-induced inhibition of invasion, and doxorubicin induced an almost complete recovery from inhibition. However, FFA and chlorpromazine had hardly any effect on the quercetin-induced inhibition. None of these PKC activators or inhibitors themselves affected cell invasion. Quercetin also significantly inhibited the migration of B16-BL6 cells, whereas none of the PKC activators or inhibitors interfered with the migration of B16-BL6 cells inhibited by quercetin (data not shown).

Effect of quercetin on the gelatinolytic activity of MMPs

B16-BL6 cells (1×10^{7}/ml) were incubated with quercetin at concentrations in the range 3.3×10^{-4} to 3.3×10^{-1} m*M* for 10 h. Only latent proforms of gelatinase A (pro-MMP-2, 72 kDa) and gelatinase B (pro-MMP-9, 92 kDa) were detected in the culture supernatants by the zymography assay. As shown in Fig. 2, quercetin markedly decreased the gelatinolytic activity of pro-MMP-9 secreted by B16-BL6 cells in a dose-dependent manner. The compound also produced a slight inhibition of the gelatinolytic activity of pro-MMP-2 (Fig. 2).

Effect of doxorubicin and chlorpromazine on the quercetin-induced inhibition of the gelatinolytic activity of pro-MMPs

B16-BL6 cells (1×10^{7}/ml) were incubated with quercetin at concentrations in the range 3.3×10^{-3} to 3.3×10^{-1} m*M* in combination with 8.6 μ*M* doxorubicin or 14 m*M* chlorpromazine for 10 h. As shown in Fig. 3, quercetin markedly inhibited the gelatinolytic activity of pro-MMP-9, but not that of pro-MMP-2. Doxorubicin produced complete blockage of the inhibition at various concentrations of quercetin (Fig. 3A, C), while chlorpromazine did not (Fig. 3B, D). Doxorubicin and chlorpromazine themselves hardly affected the gelatinolytic activities of the pro-MMPs (Fig. 3).

Quercetin inhibition of the gelatinolytic activities of MMPs enhanced by PDB and FFA

B16-BL6 cells (1×10^{7}ml) were incubated with 100 n*M* PDB or 14 m*M* FFA in the presence or absence of quercetin at 3.3×10^{-3}, 3.3×10^{-2} or 3.3×10^{-1} m*M* for 10 h. The gelatinolytic activities of MMPs were determined in the culture supernatant. Both PDB and FFA markedly increased the gelatinolytic activity of pro-MMP-9, but not that of pro-MMP-2, secreted by B16-BL6 cells (Fig. 4A lane 2, B lane 2, C, D). Quercetin dose-dependently inhibited the increases in gelatinolytic activity of pro-MMP-9 (Fig. 4A lanes 3–5, B lanes 3–5, C, D).

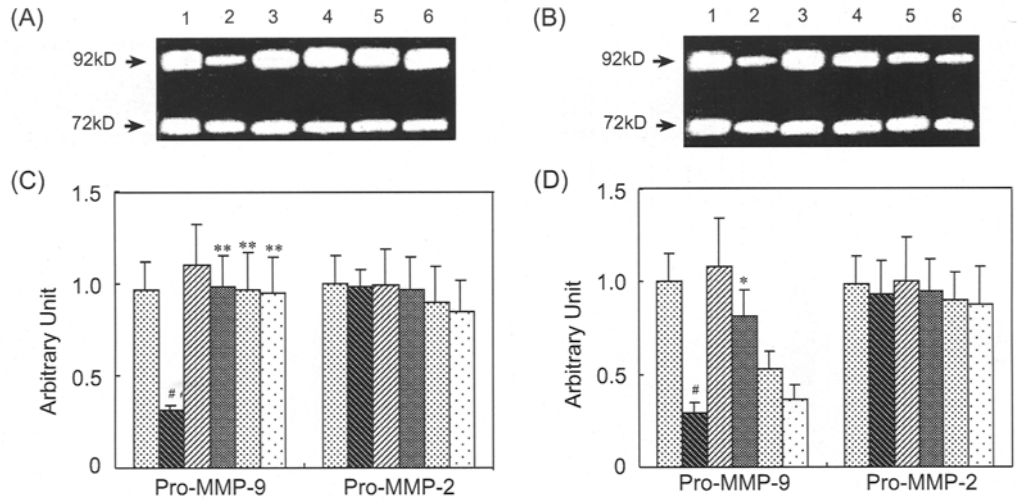

Fig. 3A–D Effect of PKC inhibitors on the quercetin-induced inhibition of pro-MMP gelatinolytic activity. B16-BL6 cells were incubated in the presence of doxorubicin or chlorpromazine with various concentrations of quercetin for 10 h at 37°C. The activity of MMPs in the supernatants was determined by gelatin zymography. **A** *Lane 1* control; *lane 2* quercetin 3.3×10^{-1} m*M*; *lane 3* doxorubicin 8.6 μ*M*; *lanes 4–6* doxorubicin 8.6 μ*M* + 3.3×10^{-3}, 3.3×10^{-2} and 3.3×10^{-1} m*M* quercetin, respectively. **B** *Lane 1* control; *lane 2* quercetin 3.3×10^{-1} m*M*; *lane 3* chlorpromazine 14 m*M*; *lanes 4–6* chlorpromazine 14 m*M* + 3.3×10^{-3}, 3.3×10^{-2} and 3.3×10^{-1} m*M* quercetin, respectively. These results are representative of three independent experiments. **C, D** Relative gelatinolytic activities of pro-MMP-9 and pro-MMP-2 were quantified densitometrically using LabWorks 4.0 software in relation to the activity without quercetin (control band assigned a value of 1). The results are expressed as the means ± SD of three independent experiments. One-way ANOVA revealed a significant effect at $P<0.01$. $^{\#}P<0.01$, vs control; $^{*}P<0.05$, $^{**}P<0.01$, vs quercetin 3.3×10^{-1} m*M* alone (Student's two-tailed *t*-test). *Six bars from left to right, respectively:* (*1*) control, (*2*) quercetin 3.3×10^{-1} m*M*, (*3*) doxorubicin 8.6 μ*M* (**C**) or chlorpromazine 14 m*M* (**D**), (*4*) doxorubicin 8.6 μ*M* + quercetin 3.3×10^{-3} m*M* (**C**) or chlorpromazine 14 m*M* + quercetin 3.3×10^{-3} m*M* (**D**), (*5*) doxorubicin 8.6 μ*M* + quercetin 3.3×10^{-2} m*M* (**C**) or chlorpromazine 14 m*M* + quercetin 3.3×10^{-2} m*M* (**D**), (*6*) doxorubicin 8.6 μ*M* + quercetin 3.3×10^{-1} m*M* (**C**) or chlorpromazine 14 m*M* + quercetin 3.3×10^{-1} m*M* (**D**)

Discussion

Quercetin (20 μ*M*), a bioflavonoid widely distributed in a variety of plants, inhibits proliferation and induces apoptosis of pancreatic tumor cells by blocking the activity of the epidermal growth factor receptor tyrosine kinase [30]. The proliferation of HL-60 leukemia cells is inhibited by quercetin at 10 to 80 μ*M* [31]. In our previous study, we found that quercetin inhibits the invasion of melanoma B16-BL6 cells by inducing apoptosis by decreasing Bcl-2 expression [28]. Quercetin also inhibits angiogenesis in vitro [32]. Furthermore, quercetin at doses from 60 to 1700 mg/m^2 in a phase I clinical trial has been shown to inhibit kinase activity and to display good safety [33].

To add to the above results and the direct inhibition by quercetin of PKC activity [34], the effects of PKC activators and inhibitors on the activity of quercetin were investigated in the present study. The PKC activators, PDB and FFA, and the inhibitors, doxorubicin and chlorpromazine, were used to interfere with the inhibition by quercetin of invasion and mobility of B16-BL6 cells. PDB significantly antagonized the quercetin-induced inhibition of cell invasion, and doxorubicin almost completely blocked inhibition. In contrast, FFA and chlorpromazine hardly affected the quercetin-mediated inhibition. Moreover, the antagonism by PDB seemed to be stronger than that by doxorubicin (Fig. 1). However, neither PDB or FFA nor doxorubicin or chlorpromazine affected the mobility of B16-BL6 cells inhibited by quercetin (data not shown). These results indicate the involvement of different mechanisms in the invasion and migration processes of B16-BL6 cells, and the inhibition by quercetin of the B16-BL6 cell invasion may be related to the PKC signaling pathway.

The PKC family has three subtypes, and the different subtypes play different or even adverse roles in tumor metastasis. For example, Powell et al. have found that overexpression of PKC-ξ inhibits the invasive and metastatic abilities of prostate cancer cells [35], and Kiley et al. have reported that PKC-δ is highly expressed in metastatic cancer cells 13762NF and that the inhibition of its activity leads to a decrease in metastasis [36]. Thus, it is also possible that the different subtypes of PKC are involved in the metastasis of B16-BL6 cells. The involvement of different PKC subtypes may explain why both PDB and doxorubicin blocked quercetin-inhibited invasion by B16-BL6 cells. However, further investigation is needed in this area.

On the other hand, the role of PKC in metastasis is closely associated with its regulation of the expression of molecules such as MMP, integrin, selectin, and CD44, which are known to play a role in tumor metastasis. The expressions of these molecules may be elevated by PKC [37, 38, 39, 40]. For example, bryostatin-1 inhibits tumor metastasis by regulating the activity of PKC that modulates the expression of MMPs [22]. To further elucidate the involvement of PKC, in the next experiment the

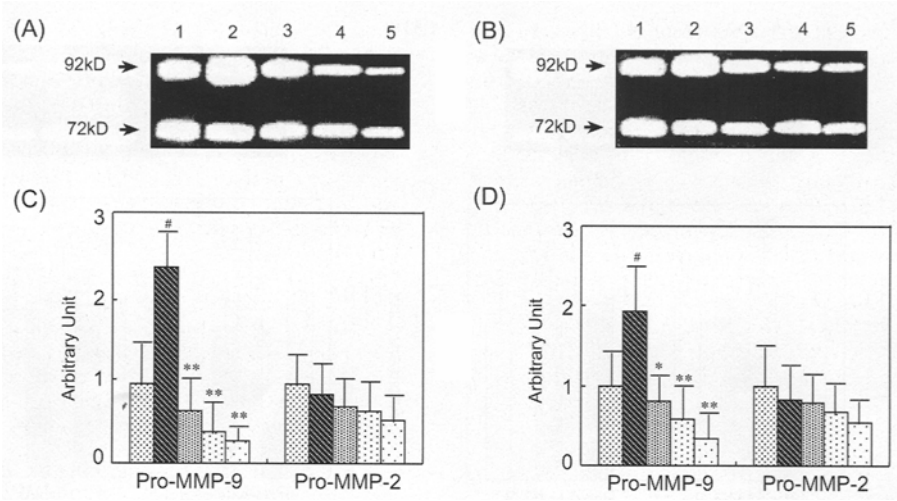

Fig. 4A–D Effect of quercetin on the gelatinolytic activity of pro-MMPs elevated by PKC activators. B16-BL6 cells (1×10^7/ml) were incubated with PDB or FFA in the presence or absence of various concentrations of quercetin for 10 h at 37°C. The gelatinolytic activity of pro-MMPs in the supernatants was determined by gelatin zymography. **A** *Lane 1* Control; *lane 2* PDB 100 n*M*; *lanes 3–5* PDB 100 n*M* + 3.3×10^{-3} m*M*, 3.3×10^{-2} m*M* and 3.3×10^{-1} m*M* quercetin, respectively. **B** *Lane 1* control; *lane 2* FFA 6.7 m*M*; *lanes 3–5* FFA 6.7 m*M* + 3.3×10^{-3}, 3.3×10^{-2} and 3.3×10^{-1} m*M* quercetin, respectively. **C, D** The relative gelatinolytic activities of pro-MMP-9 and pro-MMP-2 were quantified densitometrically using LabWorks 4.0 software in relation to the activity without quercetin (control band assigned a value of 1). The results are expressed as the means ± SD of three independent experiments. One-way ANOVA revealed a significant effect at $P<0.01$. $^{\#}P<0.01$, vs control; $^{*}P<0.05$, $^{**}P<0.01$, vs PDB 100 n*M* alone (**C**), vs FFA 6.7 m*M* alone (**D**) (Student's two-tailed *t*-test). *Five bars from left to right, respectively*: (*1*) control, (*2*) PDB 100 n*M* (**C**) or FFA 6.7 m*M* (**D**), (*3*) PDB 100 nM + quercetin 3.3×10^{-3} m*M* (**C**) or FFA 6.7 m*M* + quercetin 3.3×10^{-3} m*M* (**D**), (*4*) PDB 100 n*M* + quercetin 3.3×10^{-2} m*M* (**C**) or FFA 6.7 m*M* + quercetin 3.3×10^{-2} m*M* (**D**), (*5*) PDB 100 n*M* + quercetin 3.3×10^{-1} m*M* (**C**) or FFA 6.7 m*M* + quercetin 3.3×10^{-1} m*M* (**D**)

effects of quercetin on the activities of MMPs and the modulation by the PKC inhibitors and activators of the effects of quercetin were investigated. As shown in Fig. 2, quercetin markedly inhibited the gelatinolytic activity of pro-MMP-9 in a dose-dependent manner and slightly reduced the gelatinolytic activity of pro-MMP-2, suggesting a relationship between its antimetastatic potential and the inhibition of pro-MMP-9. Indeed, batimastat (BB-94) and marimastat (BB-2516), two specific inhibitors of MMP, which have undergone clinical trials, have been found to reduce tumor metastasis [9]. Inostamycin, an inhibitor of cytidine 5′-diphosphate 1,2-diacyl-*sn*-glycerol (CDP-DG, inositol transferase), has been found to suppress invasion of HSC-4 tongue carcinoma cells by reducing the gelatinolytic activities of pro-MMP-9 and pro-MMP-2 [41].

Moreover, the PKC activators PDB and FFA enhanced the secretion of pro-MMP-9 from B16-BL6 cells, but the ability of PDB to promote secretion of pro-MMP-9 was much higher than that of FFA (Fig. 4A lane 2, B lane 2), and quercetin also dose-dependently inhibited the enhancement (Fig. 4A lanes 3–5, B lanes 3–5, C, D). Similarly, doxorubicin, a PKC inhibitor, almost completely reversed the inhibition of pro-MMP-9 by various concentrations of quercetin, but the inhibitor chlorpromazine did not (Fig. 3A lane 4). These results are quite in accordance with the results that both PDB and doxorubicin significantly antagonized the quercetin-inhibited invasion of B16-BL6 cells (Fig. 1), and demonstrated that quercetin might inhibit invasion by reducing pro-MMPs, and more specifically by directly inhibiting the activities of PKC that modulates the expression or activity of MMPs. Interestingly, both PDB and doxorubicin regulate the activity of PKC via interaction with DAG, while both FFA and chlorpromazine regulate the activity of PKC via modulation of cytoplasmic free Ca^{2+} [16, 17, 18, 19, 20, 21], which suggests that quercetin may exerts these actions by affecting the interaction between PKC and DAG. In addition, doxorubicin also partly blocked the apoptosis of B16-BL6 cells induced by quercetin (data not shown).

In summary, both PDB and doxorubicin antagonized quercetin-induced inhibition of B16-BL6 cell invasion, suggesting the involvement of the PKC pathway. Pro-MMP-9 may be an important target in the regulation of the pathway mediated by quercetin. That is, quercetin may inhibit cell invasion by decreasing pro-MMP-9 via the PKC pathway. Further investigation is in progress to elucidate in detail the involvement of PKC subtypes.

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