Abstract. Expression and activity of CC motif ligand 2 (CCL2) is down-regulated by curcumin, the active phytochemical ingredient of turmeric (Curcuma longa), a dietary supplement often self-prescribed to promote prostate health. CCL2 is a potent chemotactic factor of prostate cancer (PCa) with important roles in development of bone metastasis. The relationship between CCL2 and curcumin, however, has not been studied in PCa. Adhesion, invasion and motility of PC-3 cells were measured in response to exposure to curcumin (30 μM; 18 h), CCL2 (100 ng/ml; 18 h) or PMA (100 ng/ml; 18 h). CCL2 mRNA expression and protein secretion levels were measured by real-time PCR and ELISA respectively. Curcumin significantly blocked CCL2 induced adhesion, invasion and motility. Curcumin also significantly suppressed the mRNA expression and secreted CCL2 protein levels. The addition of PMA, a protein kinase C (PKC) activator, blocked the effects of curcumin, leading to an increase in CCL2 expression as well as an increase in PC-3 cell adhesion, invasion and motility. The introduction of a PKC inhibitor, however, blocked the effects of CCL2. We also found that curcumin, CCL2 and PMA, in part, function through the differential regulation of the proteolytic protein matrix metalloproteinase (MMP)-9. These data indicate a potential mechanism; by which curcumin can block the chemotactic effects of CCL2 on PCa. Curcumin exerts potential anti-metastatic effects in bone-derived PCa cells by blocking CCL2 mediated actions on invasion, adhesion and motility, in part through differential regulation of PKC and MMP-9 signaling.

Introduction

The most common site of prostate cancer (PCa) metastasis is the bone, with skeletal metastases identified at autopsy in up to 90% of patients dying from PCas (1). Skeletal metastases in PCa patients result in significant complications which diminish the patient's quality of life. These include bone pain, diminished mobility, pathologic fracture, spinal cord compression, and symptomatic hypocalcaemia. Current therapeutic approaches to the treatment of prostate bone metastases have largely remained ineffective (2,3). The underlying mechanism of bone metastasis is generally poorly understood, involving complex yet aberrant crosstalk signaling between the primary tumor and the bone (2,3).

CC motif ligand 2 (CCL2; aka monocyte chemoattractant protein-1 or MCP-1) is a potent chemotactic factor vital in the development and progression of PCa bone metastasis (4-6). CCL2 is a member of the CC subfamily of low molecular weight chemokines, a class of proteins that are essential in immune regulation, inflammation, and the healing response (7). Under aberrant pathological conditions, CCL2 has essential roles in the infiltration of tumor associated macrophages, which play a key role in increased tumorigenicity of PCa and other cancers (8). CCL2 acts as an autocrine and paracrine mitogenic and motogenic factor of the malignant human androgen-independent prostatic PC-3 cell line (6). Additionally, increased expression of CCR2, the G-protein coupled receptor of CCL2, correlates with increased PCa progression (9).

CCL2 expression and activity can be inhibited by curcumin, the active polyphenolic compound of the powdered turmeric (Curcuma longa) root (10), although this relationship has never been studied in connection to PCa. Curcumin is a commonly used spice of India and Southeast Asia and is a popular dietary supplement due to its potent anti-inflammatory and anti-oxidant properties (10-12). Curcumin has shown potential as a natural low-toxicity self-treatment for benign prostatic hyperplasia (BPH) and PCa (13).

In the present study, we tested the hypothesis that curcumin inhibits CCL2 induced adhesion, invasion, and motility of PC-3 cells, a human bone-derived androgen-independent PCa cell line. We found that cells treated with curcumin exhibited decreased expression of CCL2 protein and mRNA levels, which was caused, in part, through the inhibition of the protein kinase C (PKC) signaling pathway. The PKC pathway is a central intracellular signaling mediator, with important roles in invasion and is known to up-regulate CCL2 expression.
We also found, that curcumin, through inhibition of PKC blocks CCL2-induced invasion, adhesion and motility, leading to important changes in the activity of matrix-metalloproteinase protein (MMP)-9, an important invasion related protein in PCa.

Materials and methods

Cell culture. Human derived PC-3 prostate cancer cells were maintained and grown in RPMI-1640 (Invitrogen; Carlsbad, CA) cell culture media supplemented with 10% filter sterilized fetal bovine serum (FBS) and incubated at 37°C at 5% CO2. In some experiments cells were cultured for 18 h in cell media that also contained various reagents purchased from Sigma (St. Louis, MO) including curcumin (cur; 30 μm), 1'-[2-[4-[trifluoromethyl]phenyl]ethyl]-spiro[4H-3,1-benzoxazine-4,4'-peperidin]-2(1H)-one (CCR2i; 10 μg/ml), phorbol 12-myristate 13-acetate (PMA; 100 ng/ml), Go6976, (GO; 2 μM). Some cells were also cultured with CCL2 (100 ng/ml) purchased from R&D Biosystems (Minneapolis, MN). Control cultures were treated with vehicle only. Cells were harvested at 70-80% confluency.

Quantitative real-time PCR. Total RNA was extracted using Tri-reagent from Applied Biosystems Inc. (ABI, Foster City, CA). First strand cDNA synthesis was performed using 500 ng of total RNA using random hexamer primers and SuperScript III (Invitrogen). Inventoried TaqMan real-time PCR primers for CCL2 and glyceraldehyde 3-phosphate (GAPDH) were obtained from ABI. PCR was performed on the 7500 FAST real-time PCR system (ABI) using TaqMan Universal PCR master mix (ABI). The cycling conditions for the real-time PCR: 1x (50°C for 2 min), 40x [95°C for 15 sec, 60.0°C for 1 min]. Using the relative ΔΔCT method as described by ABI, CT levels for CCL2 were normalized to GAPDH to obtain the ΔCT value. The ΔCT value was normalized to the control treatment to obtain the ΔΔCT. ΔΔCT value, in turn, was used to determine the relative expression of CCL2.

Adhesion assay. Cellular adhesion was determined as described previously with a few modifications (17). Fifty thousand cells were suspended in serum-free media supplemented with 0.1% bovine serum albumin (BSA) and added to the wells of a 48-well plate that were coated with 20 μg/ml fibronectin, collagen, or laminin. The plates were incubated for 30 and 60 min after which the plates were washed twice with PBS to remove unattached cells. Attached cells were fixed with methanol and stained with Eosin Y (Sigma). Attached cells were counted at five random optical fields (40x) as determined by light microscopy. Experiments were performed in triplicate wells and the results were averaged. Results are presented as a percentage of cellular adhesion in the presence of curcumin, CCL2, CCR2i, PMA, GO compared to cells grown with vehicle controls. The number of cells adhering under control conditions was assigned the threshold value of 100%.

Gelatin zymography. The proteolytic enzyme activity of pro-MMP-9 was measured by gelatin zymography (18). Briefly, protein concentrations were determined with the Bio-Rad DC (Bio-Rad Laboratories; Hercules, CA) protein assay kit from conditioned media and cell-lysate of PC-3 cells cultured under growing conditions as described previously (18). Total protein (15 μg) was mixed with non-denaturing buffer (125 mM Tris-HCl, 4% SDS, 20% glycerol, 0.004% bromophenol blue) and the protein samples were subjected to electrophoresis through a 10% Zymogram Ready Gel (Bio-Rad Laboratories). After electrophoresis, the gels were rinsed with wash buffer (50 mM Tris-HCl; 5 mM CaCl2, 2.5% Triton X-100) for an hour, in six 10-min intervals. After the washes, the gels were incubated overnight in incubation buffer (mM Tris-HCl; 5 mM CaCl2) at 37°C. The gels were then stained with 0.2% coomassie brilliant blue for 30 min and then destained with (30% methanol, 1% glacial acetic acid) with 3-5 washes.
Statistical analysis. Adhesion data were analyzed with Two-way ANOVA followed by Bonferonni’s post hoc comparison. Comparisons for select data groups were graphically presented. Motility, migration, CCL2 expression and zymography data were analyzed with One-way ANOVA followed by Tukey’s post hoc comparisons. Statistical comparisons were performed using GraphPad Prism Version 4.00 for Windows (GraphPad Software, San Diego, CA).

Results

Curcumin blocks the effects of CCL2-induced PC-3 adhesion, motility and invasion. PC-3 cells exposed to CCL2 exhibited a significantly higher level of adhesion to collagen, laminin, and fibronectin, when compared to controls. Curcumin blocked this effect (Fig. 1A-C). Exposure to curcumin alone, significantly decreased adhesion of PC-3 cells to fibronectin when compared to vehicle-treated controls, but not to collagen or laminin (Fig. 1A-C). Curcumin alone also inhibited the pro-motile and pro-invasive characteristics of PC-3 cells, and blocked the increased motility and invasion induced by exogenous CCL2 (Fig. 1D and E). Furthermore, the addition of the CCL2 receptor (CCR2) antagonist, CCR2i, closely mimicked the effects that were observed with curcumin; inhibiting the pro-adhesive, pro-motile, and pro-invasive effects of CCL2.

Curcumin blocks the effects of PKC-induced PC-3 adhesion, motility and invasion. In cells exposed to the PKC activator, PMA exhibited a significant increase of adhesion, motility and invasion compared to vehicle-treated controls, in a pattern similar to CCL2 (Fig. 2). Curcumin significantly
blocked these effects. Treatment with the PKC inhibitor GO blocked CCL2-induced adhesion, invasion, and motility similar to the effects of curcumin, but unlike curcumin did not alter adhesion, motility, or invasion in vehicle-treated cells (Fig. 3).

**Curcumin decreases CCL2 expression and secretion.** PC-3 cells that were exposed to curcumin, exhibited significantly lower CCL2 mRNA levels as well as significantly lower levels of CCL2 protein secretion into the media compared to vehicle-treated cells grown in the absence of curcumin (Fig. 4). We also found that PC-3 cells grown in the presence of PMA exhibited increased CCL2 mRNA expression and secretion. Curcumin treatment blocked both of these effects. The addition of GO alone did not have a significant effect on CCL2 mRNA expression and secretion, but did significantly block the increases induced by PMA (Fig. 4). GO was more effective in blocking PMA-induced mRNA expression than PMA-induced CCL2 secretion.

**Curcumin blocks CCL2-induced MMP-9 expression.** MMP-9 activity was measured in cell media of PC-3 cells by a gelatin zymography (Fig. 5A). Cells incubated with CCL2, exhibited significant 64% increase in MMP-9 activity when compared to vehicle-treated controls. Curcumin blocked these effects, but had no effect on vehicle-treated control cells (Fig. 5B). Curcumin also significantly inhibited the 75% increase of MMP-9, observed in cells incubated with PMA. There was no significant change among the different groups in relation to MMP-2 levels (Fig. 5C).
Figure 3. GO (2 μM) inhibits the adhesive, motile and invasive effects of 30 μM CCL2 on PC-3 cells. Adhesion assays were performed on fibronectin (A), collagen (B), or laminin (C) using either 30 min (black bars) or 60 min (white bars) incubations. The motility (D) and invasion (E) assays were performed using a transwell system incubated for 18 h in the absence and presence of matrigel, respectively. The results are presented as the percentage of vehicle-treated control. Each bar represents the mean ± SEM of 2-3 independent experiments. See Materials and methods for statistical analysis. *p<0.05 compared to vehicle-treated control cells. †p<0.05 compared to CCL2-treated cells. Abbreviations: GO, GO6976; CCL2, CC motif ligand 2.

Figure 4. Curcumin decreases control and PMA-induced CCL2 mRNA expression and protein secretion. PC-3 cells were exposed to curcumin (30 μM), PMA (100 ng/ml), and/or GO (2 μM) for 18 h. (A) Changes in mRNA levels of CCL2 were measured by relative real-time PCR with TaqMan Universal PCR Master Mix. Expression of target gene mRNA levels was normalized to GAPDH mRNA. (B) Changes in CCL2 protein expression/secretion was measured by a human CCL2 ELISA Ready-Set-Go! kit. The results are presented as the percentage of vehicle-treated control. Each bar represents the mean ± SEM of 2-3 independent experiments. See Materials and methods for statistical analysis. *p<0.05 compared to vehicle-treated control cells. †p<0.001 compared to CCL2-treated cells. Abbreviations: cur, curcumin; CCL2, CC motif ligand 2; PMA, phorbol 12-myristate 13-acetate; GO, GO6976.
Discussion

PC-3 cells exposed to curcumin (30 μM) alone for 18 h; a concentration and timepoint that were less than necessary to induce cytotoxicity (data not shown), exhibited a significant decrease in adhesion to fibronectin and collagen, but these cells exhibited only a moderate non-significant inhibitory trend of adhesion to laminin.

Cells exposed to curcumin also exhibited a modest, yet significant effect on invasion and motility. This finding is supported by other published reports. Hong et al for example, showed that curcumin decreased PCa tumor and metastasis burden of DU145 xenografts implanted into SCID mice, in part through the inhibition of MMP proteins after 24-h exposure (19). Additionally, they found that at increasing concentrations of curcumin (1, 5, 10 μg/ml), the invasive characteristics of cultured DU145 cells were blocked in a dose-dependent manner, with highest concentration inhibiting invasion by 71%. While, the 30-μM curcumin concentration used in our study was similar to the highest and most effective dose used in the study of Hong et al (10 μg/ml or ~27 μM), we observed the effect of curcumin in our study was far more subtle (19). We found that curcumin (30 μM; 18 h) blocked the invasive characteristics of PC-3 cells by 23% of control cells. Furthermore, we did not observe any significant effects on invasion at lower concentrations of curcumin (1, 3, or 10 μM) (data not shown).

There are several possible reasons that might explain the differences between these studies. Curcumin, for example can have very different effects in different cell types. We found that PC-3 cells exposed to higher concentrations of curcumin (≥50 μM) exhibited a significant decrease in cell viability after 24-h exposure (unpublished data). However, at equivalent

Figure 5. Curcumin (30 μM for 18 h) decreases CCL2- and PMA-induced MMP-9. Secretion and activity of pro-MMP-9 and pro-MMP-2 activity was measured by gelatin zymography. The zymography gel is representative of three separate experiments. The results were determined by measuring optical density differences of the gel using the UVP and are presented as the percentage of vehicle-treated control. See Materials and methods for statistical analysis.

*A Different from control cells at p≤0.05. †Different from CCL2 at p≤0.05. ‡Different from PMA exposed cells at p≤0.05. Abbreviations: cur, curcumin; CCL2, CC motif ligand 2; PMA, phorbol 12-myristate 13-acetate; GO, GO6976.

Figure 6. Possible pathways by which curcumin regulates CCL2 invasion and adhesion of PC-3 cells. Through an inhibition of PKC, curcumin can lead to a down-regulation of CCL2 expression and MMP-9 expression, which may be due in part to differential activation of transcription factors. Since GO blocks CCL2-induced invasion, adhesion and motility, and curcumin also blocks the up-regulation of MMP-9 by CCL2, and CCR2i blocks the effects of PMA, a potential feedback loop could be in play.
curcumin concentrations and exposure times, LNCaP, a malignant PCA cell line and RWPE1, an immortalized non-tumorigenic prostate epithelial cell line, were resistant to the effects of curcumin, exhibiting no significant changes in cell viability (unpublished data). These differences in effects of curcumin can also be observed in other cell types as well (20,21).

Additionally, unlike the Hong et al study we opted to supplement curcumin in the lower well of the transwell plates rather than the upperwell of our invasion and motility assays. In doing so, we decreased the risk of undue stress on the cells in the assay, which are incubated in serum free media in the upperwells of the transwell plates. This approach was supported by our preliminary data that showed curcumin was more cytotoxic in cells grown without FBS. Our experimental approach may have provided a more moderate curcumin effect, but the risk of cytotoxicity skewing the results was also decreased.

In this study, we also found that curcumin effectively blocked CCL2 mediated adhesion to collagen, laminin, and fibronectin as well as invasion and motility of PC-3 cells. While, CCL2 is an essential regulator of the inflammatory response, it is also a potent growth and chemotactic factor of PCa. CCL2 is an essential player in the invasive and metastatic characteristics of cancer, playing a major role in the development and progression of PCa tumor growth (22). In SCID mice, Loberg et al, showed that by using a CCL2 specific neutralizing antibody, the progression of PCa can be retarded (22). Additionally, we were able to mimic the effects observed with curcumin alone or in combination with CCL2 by treating the PC-3 cells with a CCR2 antagonist (CCR2i). CCR2 is a potential biomarker of PCa progression; increased expression of CCR2 correlates with increased malignancy of the disease (22).

PC-3 cells exposed to curcumin (30 μM, 18 h) exhibited significantly decreased levels of CCL2 mRNA and secreted protein. In preliminary experiments, we did not observe significant changes in CCL2 expression at lower concentrations of curcumin in PC-3 cells, nor did we observe significant changes in CCL2 mRNA expression in LNCaP or RWPE1 cells (data not shown). Although published reports indicate changes in CCL2 mRNA expression in LNCaP or RWPE1 of curcumin in PC-3 cells, nor did we observe any effects of exogenous CCL2 on mRNA expression or protein secretion of CCL2 (data not shown), it has been reported that several various isotypes can play differential roles in the regulation of CCL2 expression and signaling. For example, the PKC-β isoform is necessary for CCL2 induced chemotaxis, while PKC-ε plays a key role in CCL2 expression (29). PMA is known to activate numerous PKC isotypes including α, β, δ, ε, η, θ, and µ (30,31) and it is likely that CCL2 regulates PKC levels and various isotypes more selectively than PMA, which could indicate a way CCL2 might utilize PKC to induce chemotaxis, but not to up-regulate CCL2 expression. Interestingly we found that GO significantly down-regulated the effects of PMA, however, the inhibition was not complete, which was particularly noticeable when measuring the changes in CCL2 expression (Fig. 4). GO is largely a PKC-α/β inhibitor (32) and it is likely that PMA is activating the pathway to a degree that GO cannot completely block. However, since we observed that GO blocks CCL2-induced invasion, adhesion, and motility; this indicates that CCL2 may largely act through the PKC-α/β pathways (26,27). Additionally, these data suggest that curcumin also likely inhibits the PKC-ε/β pathways, as well as other PKC isotypes, particularly since the effects of curcumin on PC-3 were greater than observed with GO.

These effects of curcumin may also be in part due to the differential regulation of MMP-9. As a class of proteins, MMP proteases have essential roles in the regulation of invasion, as well as important roles in motility and adhesion, through their ability to degrade extracellular matrix (ECM) proteins (33). While important in the healing response, MMP proteins are often de-regulated in cancers and can lead to increased invasion and metastasis (33). We observed, through zymography, that CCL2 increased MMP-9 activity, but not MMP-2 in PC-3 cells and this up-regulation was blocked by curcumin (Fig. 5). Although the exact effects of CCL2 on MMP-9 are not well understood and have not been studied extensively, it has been noted that CCL2 up-regulates MMP-9 in cells of the blood-brain barrier, increasing the chance of HIV infected cells to cross into the brain (34). Additionally, macrophages from CCR2-/- mice expressed less MMP-9 compared to macrophages from a CCR2+/+ mouse (35). MMP-9 also was up-regulated by PMA, which was blocked by curcumin. It has been reported that PMA/PKC signaling is an important regulator of MMP-9 expression (36).

The exact mechanism of curcumin and its relationship with PKC and MMP-9 in CCL2-induced PCA chemotaxis is not entirely understood, however it is thought that transcription factors can play an important role (Fig. 6). For example, in
human umbilical vein endothelial cells (HUVEC), CCL2 mRNA and protein levels can be regulated by NFκB and AP-1, through PKC signaling (37). NFκB and AP-1 can also be inhibited by curcumin (38,39). NFκB and AP-1 are also important transcription regulators for MMP-9 expression (36,40). Other transcription factors such as hypoxia inducible factor 1 (HIF-1α), aryl hydrocarbon nuclear transport (ARNT aka HIF-1β), and CREB have roles in the regulation of CCL2 and MMP-9 expression are also regulated by curcumin and PKC as well (38,39,41,42).

In conclusion, this study demonstrates, that curcumin blocks adhesion, invasion, and motility of the human PC-3 cell line, in part through the down-regulation of CCL2 activity via the inhibition of PKC and MMP proteins. Further research must be performed to elucidate the importance of differential PKC isoform regulation in CCL2 signaling, as well as to achieve a better understanding of this novel mechanism by which curcumin can induce chemopreventative effects against Pca.

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References


