Monocyte Chemoattractant Protein-1 Modulates Invasion and Apoptosis of PC-3M Prostate Cancer Cells Via Regulating Expression of VEGF, MMP9 and Caspase-3

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Abstract

Prostate cancer is a form of malignancy that is most likely to develop in older males, but because of the propensity to metastasize to parts of the body, particularly the bones, it can have a deleterious impact on quality of life. Recently monocyte chemotactic protein-1 (MCP-1) has been shown to play an important role in prostate cancer progression and metastasis. In this study, we aimed to investigate the mechanisms underlying its functional roles. In vitro transwell invasion assays with PC-3M prostate cancer cells demonstrated MCP-1 promotion of invasion, while annexin V-FITC and TUNEL confirmed inhibition of apoptosis. Treatment with MCP-1 further led to significant upregulation of VEGF and MMP-9 and downregulation of Caspase-3 at both mRNA and protein levels compared with untreated control (P<0.05), while siRNA mediated knockdown reversed these changes. Taken together, our results indicate important roles of MCP-1 in prostate cancer progression and metastasis and our finding of regulation of VEGF, MMP-9 and Caspase-3 expression open up new possibilities for targeted therapy.

Keywords: MCP-1 - VEGF - MMP-9 - prostate cancer - invasion - metastasis

Introduction

Prostate cancer is a form of malignancy that most likely develops in older males. It is the second leading cause of cancer death in American men. Because prostate cancer may metastasize to other parts of the body, particularly the bones, prostate cancer has a deleterious impact on the survival and quality of life for patients. In fact, about 80% of patients who die of prostate cancer develop bone metastasis (Loberg et al., 2005; Taichman et al., 2007). Therefore, investigation of molecular mechanisms underlying the metastasis of prostate cancer to bone will be important for the prevention and therapy of prostate cancer metastasis.

Monocyte chemotactic protein-1 (MCP-1)/CCL2 is an inducible CC-chemokine that binds its G protein coupled receptor (GPCR), CCR2 and plays a vital role in the recruitment and activation of monocytes during inflammation (Rossi and Zlotnik, 2000).

More recent studies have demonstrated that MCP-1 plays crucial role in the growth, invasion, and metastasis of prostate cancer (Loberg et al., 2006; 2007b; Lu et al., 2006). Therefore, in this study, we employed a highly metastatic prostate cancer cell line PC-3M as an in vitro model to characterize the effect of MCP-1 on the cellular behaviors of PC-3M and the underlying mechanism. Our results suggest that MCP-1 regulates the expression of VEGF, MMP9 and Caspase-3 to modulate the apoptosis and invasion of prostate cancer cells.

Materials and Methods

pFIVSi-MCP-1 construct

A construct was synthesized by Sangon Biotech (Shanghai, China) MCP-1-siRNA: 5′-AAAGTCACCTGCTGTTATAACTTCACCAA-3′ (sense strand) and cloned into pFIVSi-H1/U6 vector (SBI, Mountain View, CA, USA) to make pFIVSi-MCP-1 construct. Random scramble siRNA was cloned into pFIVSi-H1/U6 vector to make pFIVSi-scramble as a negative control.

Cell culture and transfection

PC-3M prostate cancer cell line was purchased from Institute of Urology, Peking University and grown in IMDM supplemented with penicillin (50 U/ml), streptomycin (50 lg/ml) and 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA) at 37°C in 5% CO2. The cells were transfected with pFIVSi-MCP-1 or pFIVSi-scramble constructs using Lipofectamine
2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instruction. For the following experiments the PC-3M cells were divided into 4 groups. Control group: untransfected and untreated; MCP-1 group: treated with 50 ng/ml MCP-1 (Sigma, Saint Louis, Missouri, USA); Si-scramble group: transfected with pFIVSi-scramble construct; and Si-MCP-1 group: transfected with pFIVSi-MCP-1 construct.

Cell invasion assay

Cell invasion assay was performed with 24 well transwell chamber (8 μm, Costa, Lowell, MA, USA) according to the manufacturer’s instruction. Briefly, different groups of PC-3M cells (5×10⁴) were plated in the upper chamber and IMDM was added in the lower chamber. Four hours latter, cells in the upper chamber were incubated with IMDM supplemented with 1% heat-inactivated fetal bovine serum and the medium in the lower chamber was changed to IMDM supplemented with 10% heat-inactivated fetal bovine serum. The cells were incubated at 37 ºC in 5% CO₂ for 24 h and the cells in the lower chamber were stained with 0.5% crystal violet for 5 min. After washing with PBS, the stained cells were counted under microscope (Olympus IX51 at 200×) in five different fields. The assay was performed in triplicate and three times independently.

Flow cytometry analysis of apoptosis

Apoptotic cells were quantified using fluorescent Annexin V binding as described previously (van Engeland et al., 1998). Briefly, different groups of PC-3M cells were collected by digestion with EDTA-free trypsin (Invitrogen, Carlsbad, CA, USA). The cell pellet was washed with PBS twice and about 1-5×10⁵ cells were resuspended in 400 μl annexin V binding buffer (BD-Biosciences, San Jose, CA, USA). The cells were stained with 5μl annexin V FITC (BD-Biosciences) for 15 min at 4 ºC in the dark. The reaction was stopped by the addition of 10μl PI and incubation for 5 min at 4 ºC in the dark. The samples were subjected to flow cytometry analysis within 1 h. The cell suspension was also analyzed under confocal microscopy (Olympus FV1000). The assay was performed in triplicate and three times independently.

TUNEL analysis of apoptosis

Apoptotic cells were detected by TUNEL staining and subsequent confocal microscopy. Different groups of PC-3M cells were collected and seeded on glass slides pretreated with aqueous 0.01% poly-L-lysine. Cells were fixed by covering with a puddle of 1% formaldehyde in PBS for 15 min. Next the slides were incubated with TdT buffer (Promega, Madison, WI, USA) at 4ºC o/n. The membrane was developed using ECL kit (Pierce, Rockford, IL, USA) at 4ºC o/n. The membrane was washed with TBST for 5 min 3 times, then incubated with secondary antibody (Cell Signaling, Danvers, MA, USA) at 4ºC o/n. The membrane was developed using ECL kit (Pierce, Rockford, IL, USA) and exposed to X-ray film. Bands on X-ray films were quantified with Image plus5.1 software.

MCP-1 quantification by enzyme-linked immunosorbent assay

Total protein was isolated from different groups of PC-3M cells. The results demonstrated that the concentration of MCP-1 (pg/μg total protein) was 63.5±11.60, 64.9±11.07, 88.1±8.45, and 44.5±11.29 for different fields. The assay was performed in triplicate and three times independently.

RT-PCR

Total RNA was extracted from different groups of PC-3M cells using TRIzol reagent (Invitrogen, USA) following the manufacturer’s protocol. cDNA was produced by reverse transcription using RT kit (Promega, Madison, WI, USA) following the manufacturer’s protocol. PCR amplification of mcp-1, VEGF, MMP-9, Caspase-3, and GAPDH was performed with Taq Master Mix (Promega, Madison, WI, USA) with cDNA synthesized from PC-3M cells. The primers used were as follows: MCP-1 tgcgcagctataggaatactca (forward) and tgttaagttctcagaggttg (reverse); VEGF atcacaagagtgttgaagttc (forward) and aggatggcttgaagttgac (reverse); MMP-9 aagctggactctttt (forward) and cactgtgctaaactcact (reverse); Caspase-3 ggggaataggtcgttgatgt (forward) and taacgagggctggtgctggtgatga (reverse); GAPDH, gcagagctgctgtgctggtgatgt (forward) and aagaatgggagttgctgttgaagtc (reverse). Amplification conditions were as follows: 5 min at 95ºC (one cycle) and 30 sec at 94ºC; 30 sec at the annealing temperature (55ºC for MCP-1 and GAPDH, 54ºC for VEGF, 52ºC for MMP-9 and 50ºC for Caspase-3); and 30 sec at 72ºC (35 cycles) and 72ºC for 5 min (one cycle).

Statistical analysis

The experiments’ data were expressed as mean (X) ± standard deviation (S). Statistical analysis was performed using SPSS17.0 software (SPSS, Inc., Chicago, IL, USA) and P<0.05 was considered significant.

Results

Evaluation of silencing of MCP-1 in PC-3M cells

To verify that pFIVSi-MCP-1 vector we constructed could mediate silencing of MCP-1 in PC-3M cells upon transfection, we performed ELISA analysis to examine MCP-1 levels in total protein extracted from different groups of PC-3M cells. The results demonstrated that the concentration of MCP-1 (pg/μg total protein) was 63.5±11.60, 64.9±11.07, 88.1±8.45, and 44.5±11.29 for different groups of PC-3M cells. The assay was performed in triplicate and three times independently.

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control group, Si-scramble group, MCP-1 group and Si-MCP-1 group, respectively (Figure 1). Compared with control group, the level of MCP-1 was significantly increased in MCP-1 group and decreased in Si-MCP-1 group (p<0.05). Thus pFIVSi-MCP-1 vector was effective in silencing MCP-1 in PC-3M cells and could be used for the following experiments.

**MCP-1 promotes the invasion of PC-3M cells**

In vitro invasion assay demonstrated that MCP-1 treatment promoted the migration of PC-3M cells into the lower chamber compared with untreated control (Figure 2 A and C). On the other hand, siRNA-mediated knockdown of MCP-1 inhibited the migration of PC-3M cells into the lower chamber compared with scramble siRNA treated cells (Figure 2 B and D). Quantitation of the cells migrated into the lower chamber showed that there were 259.4±45.54, 267.9±48.42, 482.3±55.03, and 77.2±23.93 cells in control group, Si-scramble group, MCP-1 group, and Si-MCP-1 group, respectively (Figure 2 E). Compared with control group, there was no significant difference for Si-scramble group, however, there was significant difference for MCP-1 and Si-MCP-1 group (p<0.01). Taken together, these data suggest that MCP-1 promotes the invasion of PC-3M cells.

**MCP-1 inhibits the apoptosis of PC-3M cells**

The apoptosis rate in different groups of PC-3M cells as determined by Annexin V-FITC and propidium iodide (PI) double staining was 5.4±1.12% (control group), 7.0±3.24% (Si-scramble group), 1.0±0.59% (MCP-1 group), and 40.8±3.02% (Si-MCP-1 group) (Figure 3 A). Compared with control group, the apoptosis rate in Si-scramble group was not significantly different (p>0.05). Nevertheless, the apoptosis rate was significantly higher in Si-MCP-1 group or significantly lower in MCP-1 group (p<0.01). These results suggest that MCP-1 has inhibitory effect on apoptosis of PC-3M cells. To further confirm this, we performed TUNEL assay and found that most cells in MCP-1 group were negative for TUNEL assay, which indicated that very few cells underwent apoptosis. In contrast, most cells in Si-MCP-1 group were positive for TUNEL assay in that their nucleus were condensed and exhibited green fluorescence (Figure 3 B). Quantitative analysis of TUNEL assay showed that there was no significant difference in the apoptosis rate between control group and Si-scramble group (p>0.05). But compared with control group, the apoptosis rate was significantly higher in Si-MCP-1 group or significantly lower in MCP-1 group (p<0.01) (Figure 3 C). Taken together, these data suggest that MCP-1 inhibits the apoptosis of PC-3M cells.

**MCP-1 regulates the expression of VEGF, MMP-9 and Caspase-3**

To characterize the molecular mechanisms by which MCP-1 modulate the invasion and apoptosis of PC-3M cells, we investigated the effects of MCP-1 on the expression of VEGF, MMP-9 and Caspase-3 since VEGF and MMP-9 are involved in tumor invasion and metastasis while Caspase-3 is an important regulator of apoptosis. By RT-PCR we found that the mRNA levels of VEGF and MMP-9 were significantly increased in MCP-1 group but significantly decreased in Si-MCP-1 group (p<0.05). In contrast, the mRNA level of Caspase-3 was significantly decreased in MCP-1 group but significantly increased in Si-MCP-1 group (p<0.05) (Fig. 4 A and B). As control we
Figure 3. MCP-1 Inhibits the Apoptosis of PC-3M Prostate Cancer Cells. A. Quantitative analysis of the apoptotic cells in different groups of PC-3M cells detected by Annexin V staining. Compared with control group, the apoptosis rate was significantly higher in Si-MCP-1 group or significantly lower in MCP-1 group (p<0.01). B. The apoptotic PC-3M cells in different groups were detected by TUNEL staining. Compared with control group, the apoptosis rate was significantly higher in Si-MCP-1 group or significantly lower in MCP-1 group (p<0.01). Scale bar: 10 uM

Discussion

Overwhelming evidence collected from distinct disciplines such as in vitro tissue culture studies, preclinical animal model studies, clinical histopathology, and clinical epidemiology prove that MCP-1 is crucially involved in different phases of cancer development. As a major chemokine MCP-1 can induce the recruitment of macrophages into various human tumors ranging from bladder cancer, cervix cancer, ovary cancer, lung cancer to breast cancer (Conti and Rollins, 2004). In breast cancer, MCP-1 is highly expressed by breast tumor cells at primary tumor sites and there is a significant association between MCP-1 and advanced disease progression. Significantly, MCP-1 mediates many types of tumor-promoting cross-talks between the tumor cells and the tumor microenvironment and plays important role in metastasis and angiogenesis (Soria and Ben-Baruch, 2008). With regard to prostate cancer, recent studies suggest that MCP-1 could promote prostate cancer proliferation and inhibit cell death (Lu et al., 2006; Roca, Varsos and Pienta, 2008). It has been established that bone metastasis of prostate cancer is closely related with bone absorption of osteoclast (Keller and Brown, 2004; Roodman, 2004; Dougall and Chaisson, 2006; Datta et al., 2008). Notably, MCP-1 promotes prostate cancer growth and bone metastasis through recruitment of macrophages and osteoclast to the tumor site (Mizutani et al., 2009). In addition, MCP-1 promotes prostate cancer growth in the bone (Lu et al., 2009). Taken together, these studies convincingly prove that MCP-1 is implicated in the metastasis of prostate cancer to the bone.

As expected we observed that the mRNA level of MCP-1 was reduced in the MCP-1 siRNA treated PC-3M cells. However, treatment of these cells with MCP-1 protein induces the upregulation of MCP-1 mRNA. This is not surprising since it has been demonstrated that MCP-1 has autocrine and paracrine effects in various cell lines (Loberg et al., 2006; 2007a; Lu et al., 2006).

Angiogenesis is necessary for tumor progression, invasion and metastasis. As the most effective proangiogenic factor up to now, VEGF is highly expressed in prostate cancer and implicated in prostate cancer initiation and metastasis (El-Gohary et al., 2007; Fukuda et al., 2007). On the other hand, cancer invasion and metastasis involves cellular detachment, mobility through extracellular matrix and basement membrane...
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