Regulation of Monocyte Chemoattractant Protein-1 Through Angiotensin II Type 1 Receptor in Prostate Cancer

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Monocyte chemoattractant protein-1 (MCP-1/CCL2) is reported to contribute to tumor progression and is regulated by the renin-angiotensin system in hypertensive disease. In this study, we investigated the clinical outcome of MCP-1 expression in patients with prostate cancer (CaP) and the regulation of MCP-1 through angiotensin II (AngII) type 1 receptor (AT1R) in CaP. Specimens were obtained from 138 CaP patients and analyzed by immunostaining for both MCP-1 and macrophages. We investigated the regulation of MCP-1 expression through AT1R both *in vivo* and *in vitro* using three human prostate cancer cell lines: LNCaP, C4-2, and C4-2AT6. Specimens with a high Gleason score (≥ 7) and a high pathological classification ($\leq$ pT3), and those with castration-resistant prostate cancer showed significantly higher MCP-1 expression and higher macrophage infiltration than low malignant potential CaP. High MCP-1 expression in CaP correlated significantly with high prostate-specific antigen (PSA) recurrence rates. AngII induced significantly higher MCP-1 levels in C4-2AT6 than in LNCaP, whereas AT1R blockade (ARB) inhibited MCP-1 production via the inhibition of the PI3K/Akt pathway in C4-2AT6. ARB also significantly suppressed MCP-1 expression in C4-2AT6 tumors. Our study is the first to demonstrate that both high MCP-1 expression and high macrophage infiltration in CaP specimens correlate with a high PSA recurrence rate and that ARB inhibits MCP-1 expression through the PI3K/Akt pathway and blocks macrophage infiltration in castration-resistant prostate cancer. (Am J Pathol 2012, 180:1008–1016; DOI: 10.1016/j.ajpath.2011.11.027)

Prostate cancer (CaP) is one of the most common cancers and the second leading cause of cancer-related death in men in the United States.¹ Patients with advanced and metastatic CaP are treated mainly by androgen ablation. Unfortunately, in most of these patients, the tumor will acquire castration independence and then continue to be an incurable disease, termed castration-resistant prostate cancer (CRPC).² Recently, novel therapeutic strategies for malignancies have focused on targeting the surrounding host microenvironment as well as the tumor cells.³

Previous studies have demonstrated that macrophages, specifically tumor-associated macrophages, promote tumorigenesis and that there is a significant correlation between increased macrophage infiltration and poor patient prognosis in thyroid⁴ and hepatocellular cancers.⁵ On the other hand, one noteworthy report found that high macrophage infiltration in tumor islets correlates with a good prognosis in patients with non-small cell lung cancer.⁶ In CaP, it remains controversial as to whether macrophage infiltration in tumor lesions is an indicator of clinicopathological characteristics and clinical outcome.^{7,8}

Monocyte chemoattractant protein-1 (MCP-1/CCL2) is responsible for the recruitment of immune cells such as macrophages to sites of inflammation and has been shown to have a direct effect on endothelial cells in promoting angiogenesis.⁹ MCP-1 also regulates tumor growth, migration of tumor cells, and tumor angiogenesis⁹ by promoting macrophage mobilization and infiltration into the tumor bed in breast, lung, colorectal, and prostate cancers.^{9–12} More recently, in CaP in particular, MCP-1 is reported to have multiple roles, mainly through activation of the phosphatidylinositol 3-kinase/protein ki-

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nase B (PI3K/Akt) pathway in promoting CaP growth.¹³ However, the molecular mechanisms of upstream regulation of MCP-1 expression in tumors remain unknown. Some clinical studies showed that MCP-1 expression in CaP was correlated with malignant potential and tumor development of CaP.¹¹ However, since the number of the subjects was small and limited, the clinical role of MCP-1 expression in CaP still has not been studied in sufficient detail.

The effects of cancer-related inflammation and angiogenesis on malignancy and disease progression have been reviewed.¹⁴ Accumulated evidence suggests that the renin-angiotensin system participates in the regulation of cancer inflammation and tumor angiogenesis.¹⁵ It is known that angiotensin II type 1 receptor (AT1R), which is a component of the renin-angiotensin system, regulates MCP-1 as an inflammatory chemokine in hypertensive vascular disease¹⁶; however, there have not been any reports on the regulation of MCP-1 expression through AT1R in CaP.

In the present study, we examined the clinical role and regulation of MCP-1 expression through AT1R in CaP.

Materials and Methods

Antibodies and Reagents

Angiotensin II and mouse monoclonal antibody for β -actin were purchased from Sigma (St. Louis, MO). Rabbit polyclonal antibody for AT1R and NF κ B p65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Candesartan (CV11974 and TCV116) was provided by Takeda Pharmaceutical Co (Tokyo, Japan). CV11974 and TCV116 are active metabolites of candesartan and were used for the *in vitro* and *in vivo* experiments. Antibodies against pan Akt and phospho-Akt (Ser⁴⁷³), ERK1/2 and phospho-ERK1/2 (Thr¹⁸⁵/Tyr¹⁸⁹), JNK and phospho-JNK (Ser⁴⁷³), p38 and phospho-p38 (Thr¹⁸⁰/Tyr¹⁸²), and the PI3 kinase inhibitor LY294002 were purchased from Cell Signaling Technology (Danvers, MA). Recombinant human MCP-1 (rhMCP-1) and anti-human MCP-1 monoclonal antibody were purchased from R&D Systems (Minneapolis, MN). F4/80 (Cl:A3-1) antibody was obtained from Abcam (Cambridge, MA), and CD68 (Cl:PG-M1) was purchased from Dako (Glostrup, Denmark).

Clinical Samples and Clinicopathological Features

Archival paraffin-embedded sections were obtained from 138 patients with CaP diagnosed at Keio University Hospital. One hundred twenty-one patients underwent curative surgery that included radical prostatectomy for localized CaP between January 2000 and December 2003. None of the patients had received hormonal treatment before the operation. The characteristics of these patients are shown in Table 1. After radical prostatectomy, the patients were followed by serum prostate-specific antigen (PSA) level and imaging studies. PSA relapse was defined by an elevation of serum PSA levels at three

Table 1. Clinical and Pathological Characteristics of the Patients with Androgen-Dependent Prostate Cancer

Variable	Cases
Age (years)	
Mean (range)	66 $\pm$ 5 (50–75)
PSA (ng/mL)	
Mean (range)	10.9 $\pm$ 7.3 (3.9–49.9)
Pathological T classification	
$\leq$ pT2	52
<pT2	69
Gleason score	
$\leq$ 6	54
$\geq$ 7	67
Follow-up periods (months)	
Mean (range)	68 $\pm$ 31 (12–111)

consecutive measurements. The remaining 17 prostate specimens were also consecutively selected from the archival paraffin-embedded sections of patients who died of CRPC and had undergone autopsy at our hospital. The average age of the patients with CRPC was 70 years (range, 65 to 82 years). The Gleason score of all 17 samples was more than 8, and all were clinically stage IV. All of these patients had been subjected to androgen ablation. Histology of the specimens was evaluated by two independent pathologists using hematoxylin and eosin-stained slides. All study participants provided informed consent, and the study design was approved by an ethics review board.

Cell Lines and Culture

LNCaP cells and C4-2 cells were cultured in RPMI 1640 supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS), at 37°C in a humidified 5% CO₂ atmosphere. As previously reported, we additionally used C4-2AT6 (androgen-ablated treatment for 6 months) cells, which were obtained from C4-2 cells that were grown in RPMI 1640 containing 10% charcoal-stripped fetal bovine serum with 1% antibiotics, at 37°C in a humidified 5% CO₂ atmosphere.¹⁷ LNCaP is generally used as an androgen-dependent prostate cancer cell line, and C4-2 is also used as an androgen-independent prostate cancer cell line. C4-2AT6 is a C4-2 subline with additionally acquired malignant potential including androgen independency and aggressive angiogenesis.¹⁷

Murine Xenograft Prostate Cancer Model

Six-week-old male athymic nude BALB/C mice were castrated by scrotal incision under local anesthesia, and were used to create a xenograft model with C4-2 and C4-2AT6 cells. Twenty mice per group were subcutaneously inoculated with 5.0×10^6 cells suspended in 100 μ L of Matrigel (Becton Dickinson Labware; BD, Franklin Lakes, NJ). Tumor volume was calculated using the formula: tumor volume (mm³) = length $\times$ width $\times$ height $\times$ 0.52. Tumors were measured every 4 days for 20 days once the tumor volume had reached 50 to 80 mm³, which was defined as baseline. The animals were sacrificed

and the subcutaneous tumors were harvested on day 20. In the AT1R blockade (ARB) administration model, the treatment was started on day 1.

Cell Extracts and Western Blot Analysis

Whole-cell extracts were obtained using radioimmuno-precipitation assay buffer [50 mmol/L, Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS], containing protease inhibitors. Proteins were extracted from the cytoplasm and nucleus separately according to the standard methods.¹⁷ For Western blot analysis, equal amounts of protein were electrophoretically separated by SDS-PAGE and transferred to Trans-Blot nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were incubated overnight with primary antibody. They were then incubated with peroxidase-labeled secondary antibody (Dako). The signals were detected by enhanced chemiluminescence reagents (ECL Plus Western Blotting Detection System; Amersham Pharmacia Biotech, Uppsala, Sweden) and analyzed. The intensity was quantified using the LAS 4000 system (Fuji Film, Tokyo, Japan).

Immunohistochemical Staining and Scoring

Xenograft tumors were harvested and placed in fresh 10% formalin. The formalin-fixed paraffin-embedded sections (4 μ m) were deparaffinized, rehydrated, and then antigen retrieval was performed with Target Retrieval Solution (Dako) at 121°C for 10 minutes. Endogenous peroxidase was quenched. A blocking step was included using 1% bovine serum albumin in conjunction with avidin and biotin blocking solutions. The tissue sections were overlaid with the primary antibody and incubated at 4°C overnight. After incubation with a biotinylated secondary antibody, the tissue sections were incubated with avidin-biotin peroxidase complex, and color was developed with diaminobenzidine. After washing the slides, they were counterstained with hematoxylin. CD68⁺ cells and F4/80⁺ cells were counted in 10 randomly chosen within-tumor regions at a magnification of $\times 200$. We first determined immunoreactivity for AT1R and MCP-1 (percentage of immunostained cells to total carcinoma cells), and then the proportion score was assigned according to the percentage: 0, 0%; 1, 1% to 24%; 2, 25% to 49%; 3, 50% to 74%; and 4, 75% to 100%. The intensity score was estimated by observing the average staining intensity of positively stained cells: 0, none; 1, weak; 2, moderate; and 3, strong. The proportion and intensity scores were combined to obtain the expression score which ranged from 0 to 7.¹⁸ We classified the CaP cases into two groups with low (score 0 to 2) and high (score 3 to 7) expression for further statistical analyses.

MCP-1 ELISA

Cells were seeded in 60 mm² dishes and allowed to attach at 37°C in a humidified atmosphere of 5% CO₂/95% O₂. After 24 hours, the medium was replaced with serum-free medium for 24 hours, and then the cells were

incubated with conditioned medium (AngII 10⁻⁸ mol/L, or 10⁻⁷ mol/L). After 12 or 24 hours, the supernatant was collected, and MCP-1 was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Quantikine, R&D Systems). The protein level for MCP-1 was quantified by comparing its optical density to the standard curve for MCP-1 and normalizing it for the cell number.

Statistical Analysis

PSA relapse-free survival curves were constructed using the Kaplan-Meier method and were compared using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards model with stepwise forward selection to identify factors associated with PSA relapse. The independent variables were age, preoperative PSA, Gleason score, lymphovascular invasion, macrophage infiltration, and MCP-1 expression. Data are expressed as the mean value $\pm$ SEM. The level of significance was set at $P < 0.05$. Statistical analysis was performed using Statview 5.0 software (SAS Institute, Cary, NC).

Results

Correlation between MCP-1 Expression and Malignant Potential in Clinical CaP Specimens

To study the expression of MCP-1 and the infiltration of CD68⁺ macrophages in CaP, we classified clinical specimens according to the Gleason score and pathological stage, and analyzed the specimens by immunohistochemical staining. MCP-1-positive staining was observed in the tumor cells, extracellular areas surrounding neoplastic glands, and epithelial cells (Figure 1A). MCP-1 was expressed significantly higher in advanced-stage CaP with a higher Gleason score ($P < 0.001$) and pT3-4 ($P < 0.001$), and in CRPC ($P < 0.001$) than in low-stage CaP (Figure 1B). A high degree of infiltration by CD68⁺ macrophages was observed within neoplastic lesions. Advanced-stage CaP showed significantly higher infiltration of macrophages as well as higher MCP-1 expression than low-stage CaP (Figure 1, A and B). When the expression score of MCP-1 was classified into a low group (< 3 , $n = 93$) and a high group (≥ 3 , $n = 45$), CaP specimens with high MCP-1 expression showed significantly higher infiltration of CD68⁺ macrophages ($P < 0.0001$) in tumor lesions (Figure 1C).

Correlation between MCP-1 Expression in CaP Specimens and PSA Recurrence

Kaplan-Meier curves for 110 patients who had obvious and proper clinical characteristics showed that the 5-year PSA recurrence-free survival (RFS) rate was 77.0% in the low MCP-1 group ($n = 89$) versus 54.5% in the high MCP-1 group ($n = 21$) ($P = 0.017$) (Figure 1D). When the patients were classified according to macrophage infiltration into low (< 15) and high groups (≥ 15), the 5-year recurrence-free survival rate was 80.8% in the low group

Figure 1. **A** and **B:** MCP-1 expression and CD68⁺ macrophages were correlated with CaP malignant potential, including androgen independency (**A**), Gleason score (**B**) [†] $P < 0.0001$, ^{††} $P < 0.001$ versus Gleason score 5 and 6 specimens; $^*P < 0.05$, $^{**}P < 0.0001$ versus Gleason score ≥ 7 specimens, and pT classification, $^{\ddagger}P < 0.001$, $^{\ddagger\ddagger}P < 0.01$ versus $\leq T2$ specimens. Original magnification, $\times 200$. Scale bar = 100 μm . **C:** MCP-1 expression was correlated with the infiltration of CD68⁺ macrophages in CaP specimens. [†] $P < 0.0001$. **D:** Kaplan-Meier curves of PSA recurrence-free survival stratified by CD68⁺ macrophage and MCP-1 expression.

and 57.9% in the high group ($P = 0.018$) (Figure 1D). Multivariate analysis revealed that a preoperative high PSA ($15 \leq$) (risk ratio, 2.48) and MCP-1 expression (risk ratio, 2.20) were independent risk factors for PSA recurrence (Table 2).

Correlation between MCP-1 Expression in CaP Cells and Malignant Potential in Vivo and in Vitro

We investigated the expression of MCP-1 in CaP cells using LNCaP (as an androgen-dependent prostate cancer cell line).

Table 2. Univariate and Multivariate Analyses on 5-Year PSA Recurrence-Free Survival in Androgen-Dependent Prostate Cancer after Curative Surgery

	<i>n</i>	Univariate analysis		Multivariate analysis	
		%	<i>P</i> value	Hazard ratio (95% CI)	<i>P</i> value
Age			0.25		0.32
<65	37	69.6		1	
≤ 65	73	77.9		0.72 (0.35–1.51)	
PSA			0.0024		0.009
<15	95	78.6		1	
≤ 15	15	42.3		2.48 (1.03–5.97)	
GS			0.21		0.83
≤ 6	55	78.6		1	
≤ 7	55	69.1		1.10 (0.48–2.48)	
LVI			0.068		0.18
Negative	91	77.8		1	
Positive	19	54.4		1.65 (0.69–3.90)	
Macrophage			0.018		0.25
<15	82	80.8		1	
≤ 15	28	57.9		1.43 (0.61–3.32)	
MCP-1			0.017		<0.05
Low	89	77.0		1	
High	21	54.5		2.20 (1.01–4.42)	

The bold text indicates significant values ($P < 0.05$). CI, confidence interval; LVI, lymphovascular invasion.

Figure 2. **A:** AT1R and MCP-1 in CaP xenograft models were immunohistochemically stained and analyzed. Original magnification, $\times 400$. Scale bar = $50 \mu\text{m}$. **B:** *In vivo* and *in vitro*, AT1R expression and MCP-1 expression were correlated with androgen independency in CaP cells such as LNCaP, C4-2, and C4-2AT6. **C:** AT1R expression and MCP-1 expression of CaP cell lines *in vitro* were analyzed by Western blot analysis. * $P < 0.01$ versus LNCaP; ** $P < 0.01$ versus C4-2.

cer cell line), C4-2 (LNCaP subline; as a CRPC cell line), and C4-2AT6 (modified C4-2 cells; as an advanced CRPC cell line)¹⁷ *in vivo* and *in vitro*. Higher expression of MCP-1 in C4-2 (over 10-fold) and C4-2AT6 (over 20-fold) tumors than in LNCaP tumors was observed (Figure 2, A and B, $P < 0.01$). Higher expression of MCP-1 in C4-2 and C4-2AT6 cells than in LNCaP cells *in vitro* was also observed (Figure 2C). These results obtained using these three cell lines *in vivo* and *in vitro* show that there is a correlation between MCP-1 expression in clinical specimens and castration resistance in CaP.

Regulation of MCP-1 Expression through AT1R in CaP

It is known that the angiotensin II-AT1R (AngII-AT1R) axis regulates MCP-1 expression in vascular smooth muscle cells. We hypothesized that MCP-1 may be regulated through the AngII-AT1R axis in CaP. By immunohistochemistry, C4-2 tumors (over twofold) and C4-2AT6 tumors (over fivefold) showed significantly higher expression of AT1R than LNCaP tumors (Figure 2, A and B, $P < 0.01$). This was further confirmed by immunoblotting that C4-2 cells exhibited higher AT1R expression than LNCaP cells, and C4-2AT6 cells showed higher AT1R expression than C4-2 cells (Figure 2C). MCP-1 expression by AngII and CV11974 (as an ARB) was determined in CaP cell lines. MCP-1 production in C4-2AT6 cells was induced to a significantly higher degree by AngII 10^{-8} mol/L at 24 hours than in C4-2 cells, whereas CV11974 10^{-7} mol/L inhibited its production in both sublines ($P < 0.0001$, Figure 3). By contrast, MCP-1 was not significantly induced by AngII and was not inhibited by CV11974 in LNCaP cells (Figure 3).

Inhibition of MCP-1 Expression by ARB in Mouse Xenograft Models

We evaluated the inhibitory effect of MCP-1 by TCV116 (as an ARB) in C4-2AT6 xenograft models. TCV116 treatment resulted in a dramatic inhibition of subcutaneous tumor volume (Figure 4A). Next, we immunostained tissue samples using MCP-1 and F4/80⁺ macrophages, respectively. C4-2AT6 tumors treated with TCV116 exhibited significantly lower MCP-1 expression (about 45%, $P = 0.0033$) and lower macrophage infiltration (about 75%, $P < 0.001$) than the control group tumors (Figure 4, B and C).

AngII-AT1R Axis Regulates MCP-1 Expression through PI3K/Akt Signaling in C4-2AT6 Cells

It has been reported that the AngII-AT1R axis promotes breast cancer survival via the PI3K/Akt pathway,¹⁹ and that MCP-1 acts in an autocrine manner in CaP progression through the PI3K/Akt pathway.¹¹ C4-2 cells are

Figure 3. MCP-1 expression was regulated by AngII-AT1R axis in CaP cells. ELISA assay showed effects of AngII (10^{-8} mol/L) and CV11974 (10^{-7} mol/L) at 12 hours and 24 hours on CaP cells *in vitro*. MCP-1 expression in C4-2 cells and C4-2AT6 cells were significantly stimulated by AngII and were significantly inhibited by CV11974. * $P < 0.0001$ versus control group; ** $P < 0.001$ versus CV11974 not-treated group. ns, not significant. CV11974 had no effect on MCP-1 production in these CaP cell lines such as LNCaP, C4-2, and C4-2AT6.

Figure 4. Efficacy of candesartan compared with PBS in C4-2AT6 xenograft models. **A:** Tumor burden showed significant reduction by TCV116 (10 mg/kg, $n = 20$) compared with the control group ($n = 10$). * $P < 0.01$. **B** and **C:** MCP-1 expression and F4/80⁺ macrophage infiltration were significantly inhibited by TCV116, compared with the control group. * $P < 0.01$, ** $P < 0.001$. Original magnification, $\times 200$. Scale bar = 100 μm . Quantification shown in panel C.

known to have higher expression of phosphorylation of Akt (pAkt) than LNCaP cells,²⁰ and we have demonstrated that the expression of pAkt is markedly higher in C4-2AT6 cells than in C4-2 cells.²¹ Therefore, we hypothesized that the PI3K/Akt signaling pathway is a crucial factor for MCP-1 induced by AngII-AT1R signaling in CRPC.

We determined whether the AngII-AT1R axis regulates activation or phosphorylation of the PI3K/Akt signaling pathway in C4-2AT6 cells. ELISA assay showed that LY294002 significantly inhibited MCP-1 production in C4-2AT6 cells stimulated by AngII (Figure 5A). Next, C4-2AT6 cells were exposed to 10^{-8} mol/L AngII and/or 10^{-7} mol/L CV11974 for various periods of time (2 to 30 minutes). Western blot analysis revealed that AngII induced significant pAkt within 2 minutes. This phosphor-

ylation peaked at 5 minutes and persisted up to 10 minutes, and was inhibited by CV11974 at 5 minutes, although AngII and CV11974 had no definitive effect on total Akt protein during these time periods (Figure 5B). We have additionally investigated whether other AngII pathways such as MAPKs or JAK/STAT were involved in C4-2AT6 cells. AngII did not increase the phosphorylation of MAPKs (ERK1/2, JNK, and p38) and STAT3 during the time periods (see Supplemental Figure S1 at <http://ajp.amjpathol.org>).

Discussion

The present results have clearly demonstrated that MCP-1 expression in CaP is correlated with the malignant potential of CaP and the PSA recurrence rate, and that MCP-1 expression in CaP was regulated by the AngII-AT1R axis through the PI3K/Akt signaling pathway *in vitro*.

The infiltration of macrophages, or more specifically tumor-associated macrophages, presents in different types of human malignancies, and there have been numerous reports regarding the role of macrophages in malignant progression. However, there is still no clear consensus regarding the functional significance of macrophages in tumors. In CaP, only limited clinical data have been reported on macrophages that contribute to tumorigenicity and invasiveness.^{7,8,11,12} Moreover, it remains controversial as to whether macrophage infiltration is an indicator of the clinical outcome of CaP patients.^{7,8} This is one reason that it may be difficult to definitively classify subpopulations of macrophages in the tumor

Figure 5. MCP-1 expression was regulated by AngII-AT1R PI3K/Akt signaling in CRPC cells. **A:** ELISA assay showed effects of AngII (10^{-8} mol/L) and LY294002 (0.5 $\mu\text{mol/L}$) in C4-2AT6 cells. MCP-1 induced by AngII was significantly inhibited by LY294002 in C4-2AT6 cells. * $P < 0.0001$ versus control group; ** $P < 0.001$ versus LY294002 not-treated group. **B:** Induction of pAkt by AngII in C4-2AT6 cells. AngII (10^{-8} mol/L) induced pAkt expression in C4-2AT6 cells. CV11974 (10^{-7} mol/L) inhibited AngII-induced pAkt expression in the cells.

specimens. Monocytes migrate into tissues where they differentiate into resident macrophages, protect against infection, and respond to tissue injury. In cancer, some of these cells differentiate into macrophages that regulate tumor growth and angiogenesis, and thus macrophages with different functions may coexist in the tumor lesions. Counting only the number of macrophages in the tumor lesions may be insufficient to determine the clinical or pathological characteristics of the disease, and therefore, it would be necessary to investigate tumor-related chemokines as well as macrophages in the tumor lesions.

Elevated expression by both cancer and stromal cells of a number of monocyte chemoattractants has been shown to be positively correlated with increased macrophage numbers in several human tumors.¹² Specifically, MCP-1 has been demonstrated to regulate monocyte/macrophage trafficking and to be present in tumor beds, suggesting a role for this chemokine in macrophage recruitment.²² Mazzucchelli et al²³ claimed that MCP-1 is expressed by fibromuscular stroma cells and basal cells of non-neoplastic glands, but not by carcinoma cells. The data appear to contradict our findings on MCP-1 expression in CaP cases. However, their study examined only a few CaP cases ($n = 8$) without the data on Gleason score or pathological stage, and actually showed a low-level expression of MCP-1 in the cases, which was determined by calculating numbers of the labeled cells by *in situ* hybridization.²³ In the present study, we have demon-

strated that the expression score of MCP-1 increases with Gleason score, and early-stage CaP cases show low or negligible levels of MCP-1 expression. In addition, the MCP-1 expression levels in CaP cell lines depended on the degree of their malignant phenotype. Therefore, it may be possible to explain the discrepancy by the differences in the CaP samples examined, ie, their cases may have consisted of early-stage CaP cases with low MCP-1 expression. Consistent with our data in the present study, Lu et al¹¹ have reported that MCP-1 expression in the 83 CaP specimens correlated with the Gleason score and pathological stage, and that CaP cells (LNCaP and C4-2B cells) produced high amounts of MCP-1 compared to prostate epithelial cells *in vitro*.

A growing body of evidence suggests that the ability of MCP-1 to induce macrophage infiltration may provide a novel mechanism to support neoangiogenic growth of tumors, and that MCP-1 could become a target molecule for treating malignancies such as CaP.^{13,24} *In vivo*, MCP-1 small-interfering RNA in PC-3 cells (as CRPC cell line) has been shown to reduce tumor growth in a mouse model, and the treatment of mice with an anti-MCP-1 monoclonal antibody significantly suppressed the progression of prostate cancer bone metastasis in intracardiac and intratibial models.²⁴ However, it may be difficult to completely accept anti-MCP-1 therapy for clinical use for malignancies because MCP-1 is an essential factor in

Figure 6. Scheme showing that the AngII-AT1R axis regulates MCP-1 in prostate cancer cells. In early-stage CaP, various immune cells such as cytotoxic T cells and classical macrophages infiltrate the surrounding tumor cells in response to the tumor cells (A). Advanced-stage CaP acquires high AT1R expression, which induces MCP-1 expression through the PI3K/Akt signaling pathway. MCP-1 may act in an autocrine and paracrine manner via the PI3K/Akt pathway, and induces macrophage (as tumor-associated macrophages) infiltration for angiogenesis and proliferation of CaP (B).

biophylaxis mechanisms such as enhancement of humoral immunity and wound healing. Understanding the upstream molecular mechanism of MCP-1 expression in tumor lesions would be important for the regulation of tumor-related MCP-1 expression. Previous studies have identified both nuclear factor-kappa B (NF κ B) and activator protein-1 (AP-1) as essential regulators of MCP-1 transcription and its induction on tumor-necrotic factor α stimulation.²⁵ The AngII-AT1R axis is also known to be one of the regulators of MCP-1 expression via NF κ B and AP-1 in vascular smooth muscle cells.²⁶ In addition, recent studies have provided the evidence that the activation and/or expression of NF κ B is regulated by AngII in carcinoma tissues or carcinoma cells of the stomach,²⁷ pancreas,²⁸ and prostate.²⁹ Because our study showed that NF κ B was translocated into the nucleus after AngII stimulation and inhibited by ARB treatment in C4-2AT6 cells (see Supplemental Figure S2 at <http://ajp.amjpathol.org>), it seems likely that NF κ B activation by AngII stimulation may be one of the main transcriptional pathways for AngII-induced MCP-1 expression in CaP cells.

AngII, which is a vasoactive peptide with a variety of effects, induces inflammation through the production of reactive oxygen species, adhesion molecules, and inflammatory cytokines such as MCP-1.¹⁶ We have investigated whether the regulation of tumor angiogenesis through AT1R is related to the development and progression of mainly urological cancers such as renal cancer, bladder cancer, and CRPC.^{30–32} In particular, we previously reported that CRPC clearly showed higher AT1R expression and more aggressive angiogenesis than androgen-dependent prostate cancer.³² The present results suggest that ARB, which is widely and safely used to treat hypertensive disease, may contribute to the suppression of tumor growth through its inhibition of MCP-1 in CRPC.

AngII can activate multiple signaling pathways related to proliferation, such as protein kinase C, MAPK, and JAK-STAT3. Furthermore, stimulation of AT1R has been shown to trigger the PI3K/Akt, which plays a major role in a wide range of cellular processes.³³ Interestingly, it has been reported that MCP-1 acts in a paracrine and autocrine manner to stimulate CaP cell proliferation and migration via the PI3K/Akt pathway.¹¹ Our results would seem to support the possibility that one of the upstream regulators of MCP-1 by AngII is the PI3K/Akt pathway in CRPC cells. This signaling pathway in CRPC could be a novel therapeutic target, as shown in Figure 6. Although biological modulation by AT1R in tumors has not yet been elucidated, our findings indicate that the clinical application of ARB for CRPC with high AT1R expression may be promising.

In summary, our study has clearly demonstrated that MCP-1 expression in CaP is correlated with malignant potential and PSA recurrence, and that ARB had at least a partial inhibitory effect on tumor growth through the suppression of MCP-1 expression in CRPC that had acquired aggressive angiogenic properties accompanied by up-regulation of AT1R.

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