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Up-Regulation of Vascular Endothelial Growth Factor-D Expression in Clear Cell Renal Cell Carcinoma by CD74: A Critical Role in Cancer Cell Tumorigenesis

Yu-Huei Liu,* Chang-Yueh Lin,* Wei-Chou Lin,† Sai-Wen Tang,* Ming-Kuen Lai,‡ and Jung-Yaw Lin*2*

Elevation of CD74 is associated with a number of human cancers, including clear cell renal cell carcinoma (ccRCC). To understand the role of CD74 in the oncogenic process of ccRCC, we ectopically expressed CD74 in human embryonic kidney 293 cells (HEK/CD74) and evaluated its oncogenic potential. Through overexpression of CD74 in HEK293 and Caki-2 cells and down-regulation of CD74 in Caki-1 cells, we show that vascular endothelial growth factor-D (VEGF-D) expression is modified accordingly. A significant, positive correlation between CD74 and VEGF-D is found in human ccRCC tissues (Pearson’s correlation, \( r = 0.65, p < 0.001 \)). In HEK/CD74 xenograft mice, CD74 significantly induced the formation of tumor masses, increased tumor-induced angiogenesis, and promoted cancer cell metastasis. Blockage of VEGF-D expression by small interference RNA resulted in a decrease in cell proliferation, invasion, and cancer cell-induced HUVEC migration enhanced by CD74. Furthermore, we provide evidence that the intracellular signaling cascade responsible for VEGF-D up-regulation by CD74 is both PI3K/AKT- and MEK/ERK-dependent, both of which are associated with NF-κB nuclear translocation and DNA-binding activity. These results suggest that VEGF-D is crucial for CD74-induced human renal carcinoma cancer cell tumorigenesis.


Inflammation response plays an important role in innate immune system. Alteration in inflammation regulation leads to chronic inflammation, chronic infection, and physical damage, which result in cellular damage (1). When the damage occurs several inflammatory cytokines will be released to the damage microenvironment (1–3), and the interaction between immune-related cells and damaged cells will lead the damaged cells to proliferate, invade, and subsequently induce carcinogenesis (1).

Links between chronic inflammation and carcinogenesis may play an important role in renal cell carcinoma (RCC; 1–3), but they are not well understood. RCC is one of the most vascularized cancers, accounting for ~3% of human malignancies. One of its subtypes, clear cell RCC (ccRCC), makes up ~70% of RCC cases and is characterized by a high microvascular density produced by tumor-induced neoangiogenesis. Studies have revealed that loss of the von Hippel-Lindau gene causes an up-regulation of hypoxia-inducible factor activity, leading to overexpression of vascular endothelial growth factor (VEGF)-A (6). Therapies for metastatic RCC that include the use of the tyrosine kinase inhibitors as well as anti-EGFR and anti-VEGF agents (7) demonstrate minimal efficacy, prompting increased interest in discovering new therapeutic targets.

CD74 is a nonpolymorphic integral membrane protein, which has several functions in MHC class II (MHC-II)-restricted Ag presentation (8). Accumulating evidence has found that CD74 is up-regulated in various kinds of cancers, implying that CD74 may play an important role in tumorigenesis. Genomic studies and immunohistochemical analyses suggest that CD74 is involved in tumor growth and metastasis; CD74 is overexpressed in breast ductal carcinoma in situ (9), multiple myeloma (10), gastric cancer (11), thymic epithelial neoplasm (12), malignant fibrous histiocytoma (13), and ccRCC (3). In addition, CD74 is significantly overexpressed in pancreatic cancer cell lines with perineural invasion (14). Inhibiting the expression of the ligand for CD74, macrophage inhibitory factor (MIF), would decrease proliferation in a bladder cancer cell line (15). Blockage of MIF or CD74 decreases growth and invasion of DU145 prostate cancer cells (16). Cell surface CD74 initiates a signaling cascade that results in B lymphocyte proliferation and survival (17). However, the precise mechanisms of CD74 in tumorigenesis remain to be determined.

Although previous reports suggest a positive correlation between CD74 expression and oncogenesis, it remains unclear whether the up-regulation in CD74 expression is a cause or a consequence of the neoplastic transformation. To investigate these issues, we directly expressed CD74 in HEK293 cells and examined the role of CD74 in tumorigenesis. A search for gene(s) that might contribute to cell proliferation, invasion, and tumor-induced angiogenesis led us to the identification of one angiogenesis-related gene, Vegf-D, which played a crucial role in mediating the CD74-induced oncogenic functions in a ligand-dependent manner. By investigating the signal transduction pathway(s) involved in CD74-elicted VEGF-D, we demonstrated that CD74 induces NF-κB activation through the Ras/Raf/MEK/ERK and Ras/PI3K/
AKT signaling pathways. The results support the conclusion that up-regulation of CD74 contributes to tumor growth and metastasis of ccRCC.

Materials and Methods
Abs used for Western blot analysis and chemical inhibitors
Abs specific to CD74, VEGF-A, VEGF-C, VEGF-D, MIF, NF-κB p65, NF-κB p50, IκB-α, and phospho-IκB and Sp1 were obtained from Santa Cruz Biotechnology. Abs specific for CXCR1, CXCR2, and CXCR4 were purchased from R&D Systems. The MHC-II Ab was procured from Abcam. ERK1/2, phosphoERK1/2, AKT, and phosphorylated AKT Abs were obtained from Cell Signaling. Cyclin D, cyclin E, and actin Abs were obtained from Chemicon. The chemical inhibitors muramyl dipeptide (MDP) and Rapamycin were obtained from Tocris Cookson.

Patients, specimens, and immunohistochemical staining
The tumor and adjacent normal tissues were obtained from the Cancer Center Core of the National Taiwan University Hospital. Institutional Review Board approval was obtained to analyze the tissues used in the present study. Chemotherapy was not administered to any of the patients before surgery. All specimens were immediately frozen in liquid nitrogen and stored at −80°C before RNA extraction. The surgical specimens were fixed in formalin and embedded in paraffin before they were archived. For immunohistochemical staining, paraflin-embedded sections were deparafinized in xylene and hydrated in 95, 85, and 75% ethanol sequentially. Ags were retrieved by heating for 15 min with 10 mM citrate buffer (pH 6.0) in a microwave oven. The sections were incubated with 3% hydrogen peroxide to quench endogenous tissue peroxidase activity, and normal goat serum was used as the blocking agent (DakoCytomation). The sections were then incubated with CD74 mAb (1/2 dilution; BD Pharmingen) or VEGF-D polyclonal Ab (1/20 dilution; Santa Cruz Biotechnology) at 4°C overnight. Affinity-purified goat anti-mouse IgG or donkey anti-goat IgG conjugated with peroxidase (Santa Cruz Biotechnology) were used as secondary Abs. The sections were developed using the liquid diaminobenzidine-substrate chromogen system (DakoCytomation) and counterstained with hematoxylin (Muto Pure Chemicals).

Cell lines
The RCC cell lines 769P, 786O, ACHN, Caki-1, and Caki-2, and the human embryo kidney cell line HEK293, were obtained from American Type Culture Collection. HUVEC were obtained from Biosource Collection and Research Center. All cultures were maintained according to the manufacturer’s suggestions.

Creation and maintenance of CD74 stable transformants
The pcDNA3-1.0-CD74 was constructed by inserting a human full-length CD74 cDNA fragment (1-232 aa) into the pcDNA3.1‘’-His vector (Invitrogen). HEK293 cells were transfected with pcDNA3-1.0-CD74 using the Lipofectamine 2000 kit (Invitrogen). Stable transformants were selected by culturing in DMEM medium containing 1 mg/ml G418 (Sigma-Aldrich) and designated as HEK/CD74 cells. Each of the transformants was periodically checked for CD74 expression by Western blot analysis. All cultures were maintained in DMEM supplemented with 10% FBS (Falcon), 2 mM l-glutamine, 50 IU/ml penicillin, and 50 µg/ml streptomycin, in a humidified atmosphere containing 5% (v/v) CO2 at 37°C.

Real-time quantitative RT-PCR
Total RNAs were extracted using TRIzol reagent (Invitrogen) in accordance with the manufacturer’s instructions and further digested with DNase I (Promega). The integrity of the RNA was confirmed before quantitative (q) RT-PCR analysis. One microgram of RNA was reverse-transcribed in the presence of SuperScript II RT (Invitrogen) and oligo(dt) primers (Invitrogen) at 42°C for 1 h and then at 70°C for 15 min to inactivate the enzymes. Amplification of the cDNAs was performed using the SYBR Green PCR Master Mix (ABgene) and analyzed with the iCycler iQ Real-Time PCR Detection System (Bio-Rad). Primers were designed by the Beacon Designer 4 program (Premier Biosoft International) and are listed in Table I. The PCR conditions comprised an initial denaturation at 95°C for 15 min, followed by 40 cycles at 95°C for 10 s and 60°C for 45 s. A melting curve was performed to generate a melting curve for confirmation of amplification specificity. The results were normalized to GAPDH and the tumor protein, translationally-controlled 1 (TPT1), which has been reported to have minimal expression difference in all ccRCC cell lines.

Table I. Oligonucleotide sequences used in real-time qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide Sequence</th>
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<tr>
<td>CD74</td>
<td>5′-AGGCATCTCCCTCAGGGAAGA-3′</td>
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<td>VEGF-A</td>
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<td>VEGF-C</td>
<td>5′-GGAGAGGCAGGAAGAAGGAG-3′</td>
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<td>VEGF-D</td>
<td>5′-TGATGTATTGGAGCTGGAAG-3′</td>
</tr>
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<td>VEGFR-2</td>
<td>5′-CTTACACTAGTACCTTCT-3′</td>
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<tr>
<td>VEGFR-3</td>
<td>5′-CAACAGTGTACATGGAAGAAGAAG-3′</td>
</tr>
<tr>
<td>HIF1-α</td>
<td>5′-AATGTTTCGAAAGCCCCTCCCT-3′</td>
</tr>
<tr>
<td>HIF2-α</td>
<td>5′-TTCACCCGTTTGGTGAAGGAG-3′</td>
</tr>
<tr>
<td>TPT1</td>
<td>5′-GAGGTTGATTTCGAGGGTT-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-GCAGGGTTGTGCTTGTCTGCTG-3′</td>
</tr>
</tbody>
</table>

Animals
All animal experiments were conducted according to regulations approved by the Institutional Animal Care and Use Committee of College of Medicine, National Taiwan University. Female NOD-SCID mice (6–8 wk old) were obtained from the Animal Center of National Taiwan University. For tumor growth experiments, HEK/CD74 and HEK/Vector cell lines (5 × 10^5 cells each) were suspended in 250 µl of growth factor-reduced Matrigel (5 mg/ml; BD Biosciences) and inoculated s.c. into the flank of 6-wk-old NOD-SCID mice weighing 20–25 g (n = 8). Mice were measured weekly with calipers. Tumor volume was estimated using the formula (width^2) x length/2. At the end of the treatment the mice were sacrificed, and the tumors were removed, weighed, and processed for immunohistochemistry. Angiogenesis was determined by quantifying hemoglobin content in tumors as described previously (20). For metastasis assay, HEK/CD74 and HEK/Vector cell lines (1 × 10^5 cells each) were suspended in 200 µl of growth factor-reduced Matrigel and inoculated i.v. into the tail vein of 6-wk-old NOD-SCID mice weighing 20 to 25 g (n = 8). Mice were sacrificed after 12 wk. All organs were examined for the presence of metastatic cells. The metastatic colonies were counted. The lungs were removed, fixed, and embedded in paraffin for immunohistochemical analysis.

Short interference RNA (siRNA)
The validated chemically modified oligonucleotides used as siRNA for CD74 (5′-CAGCAACUGACAGAGGACAAGUGAU-3′) and the negative control siRNA were obtained from Invitrogen. VEGF-D siRNA (cat-noc no. sc-39844), MIF siRNA (cat-noc no. sc-37137), and control scramble siRNA (cat-noc no. sc-37007) were purchased from Sigma. A total of 2 × 10^6 cells were plated in a six-well plate for 24 h, and siRNA transfection was conducted using the Lipofectamine 2000 kit according to the manufacturer’s instructions (Invitrogen).

Cell proliferation assay
After treatment with or without the appropriate siRNA, HEK/CD74, HEK/Vector, or Caki-1 cells were seeded in 96-well plates at a density of 2 × 10^3 cells per well and cultured in 10% FBS containing culture medium. The medium was changed every day, and the cultures were maintained for 3 days. Viable cells were analyzed by measuring the conversion of the tetrazolium salt MTT to formazan crystals. After incubation for 4 h, the formazan crystals were solubilized with DMSO, and the products were quantified spectrophotometrically by measuring the absorbance at 590 nm with a reference wavelength of 650 nm using a multiwell scanner.

Anchorage-independent growth assay
CD74-expressing HEK293 stable cells and control cells were seeded in culture dishes that were fed in DMEM medium with 0.35% agarose and
10% FBS on top of a bed of 0.7% agarose in the same complete medium. After 3 wk tumor cell colonies were measured from six replicates per treatment with a dissecting microscope.

Cancer cell invasion assay

After treatment with or without the appropriate siRNA mentioned above, or Abs MIF (catalog no. AF-289-PB; R&D Systems), CD74 (catalog no. sc-5438; Santa Cruz Biotechnology), VEGF-D (catalog no. MAB286; R&D Systems), or nonspecific goat IgG (Vector Laboratories), HEK/CD74 and HEK/Vector cells were subjected to an invasion assay. Confluent cells were cultured with a growth factor-reduced medium for 24 h before harvesting. Harvested cells were resuspended at $1 \times 10^7$/ml in medium with 1% serum, and $5 \times 10^6$ cells were seeded into Transwell inserts (8 μm pore; Millipore) precoated with growth factor-reduced Matrigel (1 μg/ml; BD Biosciences). Complete medium was added to the lower chamber. After a 24 h incubation, invasive cells were fixed, stained, and quantified in three random fields (×100) per insert.

Cancer cell-induced HUVEC migration assay

For the HUVEC migration assay, 24-h growth factor-reduced HUVEC were resuspended in M199 with 1% serum, and $1 \times 10^5$ cells were seeded into Transwell inserts (8 μm pore, Millipore) precoated with 1% gelatin for the migration assay. The HUVEC-containing inserts were placed into a 24-well plate containing 700 μl of M199 with 1% serum and incubated for 1 h at 37°C. HEK/CD74, HEK/Vector, or Caki-1 cells with or without siRNA treatment were used as stimuli for HUVEC migration. The stimuli cells were seeded into the lower chamber at $2 \times 10^5$ cells per well and incubated overnight before the HUVEC inserts were added. The HUVEC that migrated were fixed and stained with 2% crystal violet and measured by counting the number of cells in three random fields (×100) per insert after 8 h.

Flow cytometry-based assays

Flow cytometry-based assays were modified according to a previous report (21). For cell surface MHC-II detection HEK/CD74 and HEK/Vector cells were fixed, permeabilized with 1% paraformaldehyde in PBS at room temperature, and washed twice in FACS staining buffer (HBSS containing 2% FBS). Cells ($5 \times 10^5$ cells/ml) were incubated with MHC-II mAbs (catalog no. ab55152; Abcam) for 20 min on ice and washed twice in ice-cold FACS staining buffer. The amounts of primary Ab remaining on the cell surface were identified by staining cells with Alexa Fluor 488-conjugated secondary Abs on ice. The cells were washed with ice-cold FACS staining buffer and analyzed by flow cytometry.

For recycling assays, HEK/CD74 and HEK/Vector cells were incubated with anti-MHC-II mAbs for 30 min at 37°C. The cells were then washed twice with ice-cold FACS staining buffer, incubated with excess goat anti-mouse IgG on ice to block surface-exposed primary Ab, washed again with ice-cold FACS buffer, and recultured in complete medium for various periods of time at 37°C. After incubation at 37°C, the cells were fixed and washed, and re-expressed MHC-II on the cell surface was detected by staining cells with Alexa Fluor 488-conjugated goat anti-mouse Ab on ice. Cells were analyzed by flow cytometry, and the relative levels of MHC-II recycling were expressed as a fraction of the amount of MHC-II reappear-

Chromatin immunoprecipitation (ChiP) assays

ChiP assays were modified from a previous report (22). In brief, CD74-expressing HEK293 stable cells and control cells, treated or nontreated with MEK inhibitor, PD98059, or the PI3K inhibitor LY294002, were cross-linked with formaldehyde (1% final concentration) for 10 min at room temperature before the reaction was stopped with glycine. After washing and lysing the cells, the lysate was sonicated and centrifuged, and the supernatant was used for immunoprecipitation of NF-κB p65 or p50 with a control Ab or Abs to NF-κB p65 or p50. Ab-bound complexes were recovered, and protein/DNA was eluted in 300 μl of elution buffer (1% SDS, 50 mM NaHCO3). Cross-linking was reversed by heating at 65°C for 4 h. The DNA was resuspended in 200 μl of water and treated with 40 μg of protease K at 37°C for 30 min, followed by phenol/chloroform extraction and ethanol precipitation. PCR was conducted using 100 ng of DNA as the template. The following PCR primers were used for the NF-κB promoter: forward primer 5'-GATGACATGACAC TCT-3' and reverse primer 5'-AGTTTAAAGACCTGTA-3'. The predicted size of the PCR was 140 bp.

Luciferase reporter assay

A total of $2 \times 10^5$ HEK/CD74 or HEK/Vector cells were plated in a six-well plate for 24 h. Following transfection with 0.1 μg of NF-κB-responsive luciferase reporters for 6 h, the cultures were grown in standard medium for another 18 h. For Ab treatment, $5 \times 10^5$ of NF-κB-reporter cells were plated in a 24-well-plate for 6 h and treated with MIF (catalog no. AF-289-PB; R&D Systems), CD74 (catalog no. sc-5438; Santa Cruz Biotechnology), or nonspecific goat IgG (Vector Laboratories) at a final concentration of 2 μg/ml for 48 h. For siRNA treatment, transfection of CD74 or MIF siRNA was conducted as discussed above in "Short interference RNA (siRNA)" for 48 h. At the end of the experiments, luciferase activity was measured using the Luciferase Assay Kit (Stratagene) accord-

Statistical analysis

SPSS 12.0 for Windows (SPSS Inc.) was used to analyze the data. A two-tailed paired-samples Student’s t test was used to analyze the significance of the differences in gene expression levels between ccRCC and adjacent normal kidney tissues, and a two-tailed independent-samples Student’s t test was used for statistical analysis of the comparative data from the two groups. A corre-

Results

Up-regulation of CD74 among ccRCC tissues and different human kidney or renal cancer cell lines and ccRCC tissues

To obtain gene expression profiles in ccRCC, the real time qRT-PCR technique was used to analyze CD74 mRNA expression in 40 pairs of ccRCC tumor (T)/nontumor (NT) tissues. The results were normalized to TPT1, which demonstrated minimal gene expression differences in all ccRCC tissue pairs (18). The expression of CD74 mRNA was up-regulated (expression ratio T/NT > 2.0) in 34 of 40 (85%) of ccRCC compared with the corresponding normal kidney tissues (−1.7 to 3.7 vs −3.5 to 0.8 in −ΔCtCD74T − TPT1T and −ΔCtCD74NT − TPT1NT, respectively; Fig. 1A; p < 0.001).
In addition, the expression of CD74 protein in RCC tissues was studied using immunohistochemistry. The brown color indicates CD74 localization, and the blue color reveals hematoxylin as a nuclei counterstain. The results suggest that CD74 expression is higher in tumor cells, but much lower expression is found in normal adjacent tissues (Fig. 1B).

The expression of CD74 in five renal cancer cell lines including 769-P, 786-O, ACHN, Caki-1, and Caki-2, and one human embryonic kidney cell line HEK293, was investigated. Interestingly, among these cell lines the higher metastatic cell line, Caki-1, expressed more CD74 (Fig. 1C). To study the subcellular localization of CD74 in cells, we transfected human CD74 into HEK293 cells, which have a low level of endogenous CD74 expression. The exogenous CD74 was found to be expressed in the cytoplasm, nucleus, and cell membrane, similar to the endogenous expression of CD74 in Caki-1 cells (data not shown).

**CD74 induced the up-regulation of VEGF-D**

To examine the role of CD74 in renal tumorigenesis, real time qRT-PCR analysis was performed to examine the effect of CD74 on the expression of genes. The results showed that CD74 up-regulates VEGF-D expression, but not Vegf-A or Vegf-C, in HEK293 cells (Fig. 2A). In addition, not only the 58-kDa VEGF-D precursor but also the 29/31-kDa intermediate and 21-kDa mature VEGF-D proteins were increased by CD74 (Fig. 2B). Meanwhile, real time qRT-PCR analysis revealed no significant change in the expression levels of Hif-1α, Hif-2α, VEGFR-2, and VEGFR-3 as shown by real-time qRT-PCR analysis (Fig. 2C). To confirm this observation Caki-1 and Caki-2 cells were used. Through overexpression of CD74 in Caki-2 cells as well as down-regulation of CD74 in Caki-1 cells, we demonstrated that VEGF-D protein expressions were modified accordingly (Fig. 2D).

MIF is a ubiquitously expressed cytokine that is thought to play an important role in carcinogenesis by promoting cell proliferation (23), tumor angiogenesis (23, 24), and metastasis (25, 26), which may be promoted through binding to CD74 (27). To clarify the relationship between CD74 and MIF in the induction of VEGF-D, HEK/CD74 cells were first transfected with CD74 siRNA or MIF siRNA to inhibit VEGF-D expression, and then rMIF was added to rescue the inhibition. The results showed that the rMIF can rescue the inhibition of VEGF-D by MIF siRNA, but not that by CD74 siRNA (Fig. 2E). These results indicate that CD74-induced expression of VEGF-D is ligand dependent.

**Correlation between CD74 and VEGF-D expression in ccRCC biopsy tissues**

To further investigate the correlation between CD74 and VEGF-D, real-time qRT-PCR analysis was performed to analyze mRNA expression of CD74 and VEGF-D in 40 ccRCC tissues. The results
showed that CD74 and VEGF-D expression levels in ccRCC tissues were higher than those in normal tissues. A significant, positive correlation was found between CD74 and VEGF-D (Fig. 3A; Pearson’s correlation, \( r = 0.65, p < 0.001 \)). Immunohistochemical analysis was performed to examine clinical ccRCC biopsy samples from 20 of 40 patients (Fig. 3B and Table II). After staining for CD74 or VEGF-D, we observed that CD74 was detectable in the cytoplasm, nucleus, and cell membrane of cells in 18 of 20 (90%) ccRCC tissues, whereas VEGF-D was expressed in the cytoplasm in 13 of 20 (65%) tumor samples. The adjacent nontumor tissues had little or no CD74 or VEGF-D expression. A 0–3 scoring system was used to represent the percentage of positively stained cells in tumor and nontumor portions of ccRCC tissues. Similar expression correlation between CD74 and VEGF-D was shown in kidney and RCC cell lines (Fig. 1C). The data reveal that CD74 expression correlates with VEGF-D expression.

**CD74 induces tumor growth and metastasis**

The role of CD74 in tumorigenesis was examined in immunodeficient NOD-SCID mice. The mean body weight and overall activity of mice were similar in both groups during the experiment. Tumor growth was significantly enhanced in the group injected with HEK/CD74 cells (5701.4 ± 1400.1 mm³) when measured on days 42 to 90, whereas no significant growth of the tumor was found in the group injected with HEK/Vector cells (23.4 ± 30.4 mm³; Fig. 4A; \( p < 0.01 \)). Difference of tumor weight between two groups of tumor masses was also shown (4496.0 ± 1358.8 mg vs 157.9 ± 28.4 mg in HEK/CD74 and HEK/Vector, respectively; Fig. 4B; \( p < 0.05 \)). A significant difference in hemoglobin content between the two groups of tumor masses indicated that CD74 induced angiogenesis in vivo (6.5 ± 0.9 g/dL vs 0.4 ± 0.1 g/dL in HEK/CD74 and HEK/Vector, respectively; Fig. 4C; \( p < 0.01 \)). These observations support the notion that CD74 induces tumor growth.

Because the expression of CD74 may correlate with metastatic character in cancer cell lines (Ref. 16, DU-145 cells and this study, Fig. 1C, Caki-1 cells), we further investigated the possible role of CD74 in tumor metastasis in vivo. HEK/CD74

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**Table II. Clinicopathologic feature and expression of CD74 and VEGF-D in 20 ccRCC biopsies**

<table>
<thead>
<tr>
<th>% Positive Cell (Score)</th>
<th>Positive Cases</th>
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<td>Tumor</td>
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<td></td>
</tr>
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<td>CD74</td>
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<td>18 90</td>
</tr>
<tr>
<td>VEGF-D</td>
<td>7 9 2 2</td>
<td>13 65</td>
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<tr>
<td>Adjacent tissue</td>
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<tr>
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*Total number of ccRCC patients, \( n = 20 \). Scoring system: 0, 0–10% positive staining; 1+, 11–30% positive staining; 2+, 31–60% positive staining; 3+, 60–100% positive staining.

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**FIGURE 4.** CD74 promotes tumor growth, angiogenesis, and cancer cell metastasis in vivo. **A**, Tumor volumes were measured at weekly intervals. **B**, Weights of excised tumor masses were determined at day 90 after inoculation. **C**, CD74-induced angiogenesis was determined by hemoglobin content of the tumor mass. **D**, Number of metastatic nodules was counted after lung metastasis assay. **E**, Weights of excised lungs were determined at day 90 after injection. **F**, Overexpression of CD74 did not alter the protein level, but reduced the cell surface expression of MHC-II. **Left panel,** the expression of MHC-II in HEK/CD74 and HEK/Vector cells was detected by Western blot analysis. **Right panel,** FACS analysis of surface MHC-II expression in HEK/CD74 and HEK/Vector cells. Cells were harvested and labeled with Abs against MHC-II. FITC-conjugated secondary Abs were used to detect bound MHC-II Ab. A mouse IgG2a was used as the isotype control. **G**, Overexpression of CD74 delayed the cell surface presentation of MHC-II by FACS analysis (Ref. 16, DU-145 cells and this study, Fig. 1C, Caki-1 cells), we further investigated the possible role of CD74 in tumor metastasis in vivo. HEK/CD74

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*Total number of ccRCC patients, \( n = 20 \). Scoring system: 0, 0–10% positive staining; 1+, 11–30% positive staining; 2+, 31–60% positive staining; 3+, 60–100% positive staining.
or HEK/Vector cells were injected i.v. into the lateral tail vein of NOD-SCID mice. As we expected, after 90 days the lung metastases occurred more frequently in mice injected with HEK/CD74 cells than in those injected with HEK/Vector cells (96.5 ± 26.2 vs 24.3 ± 7.7 nodules/lung, respectively; Fig. 4D; p < 0.01). Difference of weight between two groups of lungs was also shown (367.4 ± 115.3 mg vs 173.9 ± 21.0 mg in HEK/CD74 and HEK/Vector, respectively; Fig. 4E; p < 0.05). These observations support the idea that CD74 induces tumor metastasis.

Because persistent high expression of CD74 in the intracellular compartment and on the cell surface could impair MHC-II Ag presentation by tumor cells, it could contribute to immune escape and tumor metastasis (28). We examined the expression of MHC-II in response to CD74 up-regulation. Western blot analysis revealed no significant change in the expression level of MHC-II in response to CD74 overexpression (Fig. 4F, left panel). However, overexpression of CD74 suppressed cell surface MHC II expression (Fig. 4F, right panel). The kinetic recycling assay also demonstrated that CD74 expression delayed ~20% of MHC-II representation on the cell membrane (Fig. 4G). Similar results were obtained when the kinetics of MHC-II recycling were examined in monocyte-derived human DCs (results not shown). The results indicate that inhibition of MHC-II Ag presentation might be

**FIGURE 5.** CD74-induced expression of cyclin D and cyclin E as well as cell proliferation were reduced by inhibition of VEGF-D. A, Growth analysis of HEK/CD74 and HEK/Vector cells was measured by the MTT assay. The growth rate is expressed as the percentage increase in OD compared with time zero. B, Anchorage-independent growth analysis of HEK/CD74 and HEK/Vector cells was determined using the soft agar assay. C, CD74-induced overexpression of cyclin D and cyclin E. D, Inhibition of VEGF-D for 24 h reduced the expression of cyclin D and cyclin E induced by CD74 in either HEK/CD74 or Caki-1 cells. E, Silencing of VEGF-D by siRNA reduced cell proliferation of HEK/CD74 or Caki-1 cells. F and G, Inhibition of CD74 signaling by siRNA of CD74 or MIF reduced cell proliferation of HEK/CD74 or Caki-1 cells. The average ± SD is shown from at least six separate experiments. * p < 0.05, ** p < 0.01, *** p < 0.001.
VEGF-D is involved in CD74-induced promotion of cell proliferation and invasion

Our results suggest that CD74 has other functions in addition to inhibition of MHC-II presentation. Because CD74 induced the expression of VEGF-D, we turned our attention to whether VEGF-D participates in the oncogenic function of CD74. CD74 promotes cell proliferation (2.0-fold at day 3; Fig. 5A; p < 0.01) and anchorage-independent growth (3.8-fold at day 21; Fig. 5B; p < 0.05). Two regulators in G1/S phase, cyclin D and cyclin E, were reported to be up-regulated by CD74 and promote cell cycle progression (17). VEGF-D siRNA was used to examine the possible role of VEGF-D in mediating the expression of cyclin D and cyclin E. CD74 up-regulates expression of cyclin D and cyclin E in HEK293 cells (Fig. 5C). When VEGF-D was silenced with siRNA, the expression of cyclin D and cyclin E, as well as cell proliferation, were reduced accordingly in either HEK/CD74 or Caki-1 cells (Fig. 5, D and E). The results were further confirmed under blockage of expression of CD74 or MIF in either HEK/CD74 or Caki-1 cells (Fig. 5, F and G). The data show that CD74-up-regulated VEGF-D positively regulates the expression of cyclin D and E, which results in promotion of cell cycle progression.

The expression of CD74 may correlate with cell invasion (Ref. 16 and this study, Figs. 1C and 4D). In addition, VEGF-C, which is highly homologous to VEGF-D, induces lung cancer metastasis by inhibiting the expression of the cell adhesion molecule, contact-1 (22). According to these data, we examined the possible role of VEGF-D in CD74-induced invasion. CD74 enhanced Matrigel invasion (3.6-fold; Fig. 6A; p < 0.05). After silencing VEGF-D by siRNA treatment, the invasion induced by VEGF-D was reduced in both HEK/CD74 (6.0-fold; Fig. 6A; p < 0.01) and Caki-1 cells (8.7-fold; Fig. 6B; p < 0.01). Similar inhibition patterns were observed under blockage of VEGF-D Ab or blockage of CD74 or MIF with specific Ab or siRNA. These results support the hypothesis that CD74 induces tumor proliferation and invasion via up-regulation of VEGF-D.

VEGF-D is involved in CD74-induced promotion of tumor-induced HUVEC migration

Because all tumors must undergo angiogenesis or neovascularization to acquire nutrients for growth and metastatic spread (29), and VEGF are the most important inducers of angiogenesis, we investigated whether CD74-induced VEGF-D directs the angiogenesis we observed in xenograft mice. HUVEC were cocultured with HEK/CD74, HEK/Vector, or Caki-1 cells with or without VEGF-D siRNA treatment. HEK/CD74 cells resulted in a 2.5-fold increase in HUVEC Transwell migration activity compared with HEK/Vector (p < 0.01). The data suggest that CD74 promotes HUVEC migration via VEGF-D, indicating its role in promotion of angiogenesis.

It has been suggested that MIF binds to CXCR1 and CXCR4 (30), and these receptors play a role in tumorigenesis and metastasis of RCC (31–33). Therefore, we examined whether these receptors play a role in the expression of VEGF-D during CD74 up-regulation. Western blot analysis revealed no significant elevation in CXCR2 or CXCR4, but moderate expression of CXCR1, in HEK/CD74 cells (Fig. 8A). Blocking these receptors with specific Abs for 16 h demonstrated no significant change in VEGF-D expression (data not shown). However, blocking CXCR2 or CXCR4 for 48 h resulted in a decrease in CD74-induced VEGF-D expression (Fig. 8B, lanes 4–5). A slight decrease in VEGF-D expression was also observed when CXCR1 was blocked (Fig. 8B, lane 3). In addition, after blocking these receptors the CD74-VEGF-D-enhanced cell proliferation was only reduced at day 3 (~1.3-fold; Fig. 8C; p < 0.01). Decreased cell invasion was also observed when these receptors were blocked (3.0-fold, 2.3-fold, and 2.6-fold for CXCR1, CXCR2, and CXCR4, respectively; Fig. 8D; p < 0.01). These results suggest that overexpression of CD74 may not increase the expression of CXCR1, 2, and 4, but these receptors

FIGURE 6. Inhibition of CD74-up-regulated VEGF-D reduced its ability to promote cancer cell invasion. A, Measurement of cell invasion by the Boyden chamber assay. The invasive activity was significantly higher in CD74-transfected cells as compared with vector control cells. B, The invasion of HEK/CD74, HEK/Vector, or Caki-1 cells treated with or without CD74, VEGF-D, or MIF siRNA or Ab was measured. The results shown in this figure represent the average ± SD from six separate experiments. **, p < 0.01.

FIGURE 7. Inhibition of CD74-up-regulated VEGF-D reduced its ability to promote cancer cell-induced HUVEC migration. A and B, HUVEC were cocultured with HEK/CD74, HEK/Vector, or Caki-1 cells with or without treatment of CD74, VEGF-D, or MIF siRNA. The data suggest that CD74 promotes HUVEC migration via VEGF-D, indicating its role in the promotion of angiogenesis. The results shown here represent the average ± SD from six separate experiments. **, p < 0.01.

FIGURE 7.
certainly play a partial role in CD74-induced VEGF-D expression, as well as subsequent cell proliferation and invasion.

NF-κB is involved in CD74-mediated VEGF-D expression

The transcription factor NF-κB is one of the important factors activated by CD74 in human cells (17). While determining the

FIGURE 8. CXCR1, 2, and 4 are partially involved in CD74-mediated proliferation and invasion. A, CD74 does not significantly alter the expression of CXCR1, 2, and 4, as indicated by Western blot analysis. B, Blocking of CXCR1, 2, or 4 by specific Abs for 48 h reduced CD74-induced VEGF-D expression, as demonstrated by Western blotting. C, Blocking of CXCR1, 2, or 4 reduced CD74-induced cell proliferation. D, Blocking of CXCR1, 2, or 4 reduced CD74-induced cell invasion. The results shown represent the average ± SD from six separate experiments. *, p < 0.05. **, p < 0.01.

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The transcription factor NF-κB is one of the important factors activated by CD74 in human cells (17). While determining the

FIGURE 9. Activation of NF-κB is required for VEGF-D expression. A, upper panel, Immunoblotting (IB) analysis of NF-κB nuclear translocation activity. Nuclear extracts were prepared from serum-starved HEK/CD74 treated with or without 20 μM PD98059 (PD) or 25 μM LY294002 (LY) for 2 h. The nuclear extracts were subjected to Western blot analysis using the indicated Abs. A, lower panel, ChIP analysis of NF-κB DNA-binding activity. The CD74-induced DNA-binding activity was decreased by treatment with PD98059 or LY294002. B, Luciferase activity analysis of NF-κB. The NF-κB transcriptional activity was measured by the luciferase assay using an optimal NF-κB-binding site in tandem followed by luciferase. The data are representative of six independent experiments. **, p < 0.01, ***, p < 0.001. C, Dominant-negative (DN) I-κBα down-regulates CD74-induced VEGF-D expression. HEK/CD74 or HEK/Vector cells were transfected with dominant-negative I-κBα. The expression of VEGF-D in HEK/CD74 cells was down-regulated. Phosphospecific Ab recognizes I-κBα phosphorylated at serine 32.
NF-κB, demonstrating the specificity of the CD74-induced activation (Fig. 9A). Reduced when inhibition of either CD74 or MIF occurred, indicating a significant repression of NF-κB nuclear translocation and elimination of CD74-induced VEGF-D expression (Fig. 9C). These results indicate that IκB phosphorylation/degradation and subsequent NF-κB activation are required for the VEGF-D expression induced by CD74.

Ras-Raf-MEK-ERK and PI3K-AKT-mediated NF-κB signaling pathways are required for CD74-induced VEGF-D expression

Previous papers have shown that Ras/Raf/MEK/ERK and PI3K/AKT kinase signaling pathways are involved in CD74-mediated cellular function (34, 35) through association with CD44 (36, 37). In this study, we have shown that both nuclear translocation and DNA binding activities are blocked by the MEK inhibitor, PD98059, and the PI3K inhibitor, LY294002 (Fig. 9A, lanes 3–4). To study which pathway or pathways are involved in CD74-mediated VEGF-D activation, potential CD74-mediated ERK and AKT signal transduction pathways were first examined. Various inhibitors of these pathways were used to study their inhibitory effects on CD74-mediated VEGF-D activation. Manumycin A (Ras inhibitor; 2 μM), GW 5074 (Raf inhibitor; 0.5 μM), PD 98059 (MEK inhibitor; 20 μM), and LY 294002 (PI3K inhibitor; 20 μM) were used to treat HEK/CD74 cells, and we observed that the overexpression of VEGF-D was inhibited by these signaling inhibitors (Fig. 10A, lanes 3–5, 8, and 9). To examine the possibility that VEGF-D augments its own expression in CD74-expressing cells in an autocrine fashion, we blocked VEGF-D autocrine signaling by treatment with a specific neutralizing Ab. The CD74-up-regulated ERK and AKT phosphorylation was partially blocked after treatment with VEGF-D-neutralizing Ab (Fig. 10B, lanes 3–4). Our results reveal that VEGF-D may regulate its own expression in an autocrine manner, and that CD74-mediated VEGF-D expression is regulated via ERK and AKT signaling pathways.

Discussion

Several pieces of evidence have shown that CD74 is up-regulated in several types of cancers (3, 9–16). Our report also showed that CD74 is remarkably up-regulated in ccRCC tissues by full-length enriched cDNA library screening (38). However, the oncogenic potential of CD74 in ccRCC remains to be elucidated. In this study, by using real-time qRT-PCR analysis, we show that CD74 is significantly up-regulated in 40 ccRCC tissue pairs. By immunohistochemical analysis, we also demonstrate that CD74 is overexpressed in ccRCC tissues. Although it is suggested that excessive CD74 expression in malignancies might block endogenous tumor Ag presentation by MHC-II, resulting in immune escape in vivo (28), our study provides evidence for the oncogenic role of CD74 in addition to inhibition of MHC-II presentation. This is the first demonstration that increased expression of CD74 can enhance kidney cancer cell proliferation, anchorage independence, invasion, and tumor cell-induced HUVEC migration in vitro as well as tumor growth and metastasis in vivo. Our studies are consistent with other in vitro and in vivo studies showing that inhibition of CD74 expression reduces DU-145 prostate cancer cell growth and invasion (16).

Potential sources of MIF, such as macrophages and primary tumors, could direct tumor cell growth in an autocrine or paracrine loop (24, 39). The major source of MIF may be the tumor cell itself, the endothelial cell, the T cell, or the macrophage. Thus, MIF could stimulate tumor cell growth directly. Our study shows that down-regulation of MIF significantly reduces cell oncogenic functions in vitro and that addition of rMIF in HEK/CD74 cells induces more abundant VEGF-D than that in HEK/Vector cells. Despite this, the expression of MIF is not statistically different
between ccRCC and nontumor tissues (data not shown). Thus, increased CD74 expression may enhance tumor growth in response to MIF, demonstrating that the increased sensitivity due to higher levels of CD74 is in response to MIF in CD74-expressing cells.

Although the roles of CD74 and VEGFs in the pathogenesis of ccRCC have been individually discussed (6), the link between CD74 and VEGFs has not been found. Current therapeutic agents that specifically inhibit VEGF signaling (mostly VEGF-A signaling) demonstrate limited efficacy in tumor suppression of ccRCC, suggesting that blocking VEGF-A alone might not be sufficient for complete inhibition of tumor growth and metastasis. VEGF-D belongs to the VEGF family, which plays a major role in angiogenesis and lymphangiogenesis (40). Because of its potent induction of tumor growth, survival, and metastasis, VEGF-D is tightly regulated at low or undetectable levels in normal tissues. Some factors were reported to induce VEGF-D expression, such as hypoxia (40) and IL-7 (41). Our unpublished data indicated that the VEGF-D receptor, VEGFR-3, is not overexpressed but highly phosphorylated in the CD74-expressed cells, indicating that the functions induced by VEGF-D may be done through the cancer cell-expressed VEGFR-3. In this study, we demonstrate for the first time that activation of the MEK/ERK and PI3K/AKT pathways is required for CD74-mediated up-regulation of the angiogenic factor VEGF-D in human kidney cancer cells in an MIF-dependent manner. In addition, we found the activation of NF-κB by CD74 may involve in the up-regulation of VEGF-D. Mutations in the NF-κB sites in the VEGF-D promoter have to be examined to determine the contribution of NF-κB in response to CD74, because there are still several candidate transcription factors that may be involved in VEGF-D expression. Our current report on the induction of VEGF-D protein expression contributes to a better understanding of the mechanism of CD74-induced tumorigenesis.

It has been shown that CD74 promotes cell proliferation through activation of cyclin D and cyclin E expression in B lymphocytes (16, 17). Binsky et al. demonstrated that CD74-expressing B-CLL cells constitutively produce and release biologically active IL-8, which induces Bcl-2 expression, resulting in increased cell survival but not enhanced cell proliferation (42). This supports the concept that, although both IL-8 and VEGF-D are known proinflammatory factors that may regulate chronic inflammation, leading to tumor growth and angiogenesis in an autocrine manner (43), they must be tightly controlled. CXCR1, 2, and 4 appear to mediate CD74-up-regulated VEGF-D expression, but because CD74-induced VEGF-D expression is MIF dependent and CD74 can induce overexpression of IL-8, that is perhaps why blockage of CXCR receptors reduces less responses than CD74 in our experiments. Indeed, our data demonstrate that CD74 enhances cell growth and the expression of the cell cycle-regulators cyclin D and cyclin E through VEGF-D, which results in promotion of cell proliferation. Further studies will focus on discovering the target gene of CD74-mediated VEGF-D that affects cell invasion.

Cell invasion is important for cancer progression and metastasis. A functional linkage between CD74 and metastasis has been implied in earlier reports (9, 16). One of the molecular mechanisms related to the advanced angiogenic phenotype of ccRCC is loss of the von Hippel-Lindau gene, resulting in accumulation of HIF and leading to overexpression of VEGF-A (6). Despite this, in our study CD74 could not significantly induce the expression of HIF1-α or HIF2-α, suggesting that an alternative regulation mechanism exists in ccRCC metastasis. Meanwhile, reducing the expression of VEGF-D in either HEK/CD74 or Caki-1 cells reduced the CD74-enhanced cancer cell invasion. These results implied that CD74-induced VEGF-D may play a role in metastatic ccRCC.

However, the clinical significance of this finding must be investigated.

In summary, our findings identified for the first time that CD74 potently induces the up-regulation of VEGF-D mRNA and protein expression in human kidney cancer cells through MEK/ERK and PI3K/AKT signaling pathways, leading to the subsequent activation of NF-κB. Our study also provides a therapeutic rationale for the inhibition of ccRCC growth, invasion, and angiogenesis by treatment with pharmacological inhibitors to block the signaling cascade or by blocking CD74 signaling through reducing the expression of MIF, CD74, or VEGF-D. A drug targeting CD74 signaling together with other currently available therapeutic agents may serve as a promising strategy in combating tumorigenesis and metastasis in ccRCC.

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Disclosures

The authors have no financial conflict of interest.

References


