ELR⁺-CXC Chemokines and Their Receptors in Early Metanephric Development

Zoia B. Levashova,* Nirmala Sharma,* Olga A. Timofeeva,* Jeffrey S. Dome,† and Alan O. Perantoni*

*Laboratory of Comparative Carcinogenesis, National Cancer Institute, National Institutes of Health, Frederick, Maryland; and †Division of Oncology, Children’s National Medical Center, Washington, DC

ABSTRACT
Although originally identified as mediators of inflammation, it is now apparent that chemokines play a fundamental role in tissue development. In this study, ELR⁺-CXC chemokine family members CXCL2 and CXCL7, along with their preferred receptor CXCR2, were expressed at the earliest stages of metanephric development in the rat, and signaling through this receptor was required for the survival and maintenance of the undifferentiated metanephric mesenchyme (MM). A specific antagonist of the CXCR2 receptor SB225002 induced apoptosis in this population but did not affect more mature structures or cells in the ureteric bud. CXCL7 treatment of isolated MM elicited an angiogenic response by upregulation of matrix metalloprotease 9 and endothelial and mesangial markers (platelet-endothelial cell adhesion molecule, Megsin, Thy-1, PDGF receptor α, and vascular α-actin) and induced SB225002-sensitive cell invasion through a matrix. Because Wilms’ tumor cells may similarly depend on CXCR2 signaling for survival, primary tumor samples were analyzed, and 15 of 16 Wilms’ tumors were found to be CXCR2 positive, whereas grossly normal kidney tissues from tumor patients or renal cell carcinomas were CXCR2 negative. Furthermore, cell lines derived from Wilms’ tumors but not those from renal cell carcinomas were sensitive to SB225002-induced apoptosis. These data provide evidence for a prosurvival and proangiogenic role of ELR⁺-CXC chemokines and their receptor CXCR2 during metanephric development and suggest a novel mechanism for chemotherapeutic intervention in Wilms’ tumor.


Metanephric development requires mutual interactions between the ureteric bud (UB) and the metanephric mesenchyme (MM). MM induces growth and branching of the UB, whereas survival and differentiation of the MM into nephronic epithelia depends on factors secreted by the UB.¹ Nephron-inducing UB-secreted factors have been identified and include leukemia inhibitory factor and TGF-β.²,³ In a search for new UB-secreted inductive molecules, we applied microarray technology to a rat UB-derived cell line⁴ and implicated chemokines as novel participants in kidney development.

Chemokines belong to one of four families of secreted polypeptides, initially identified for their ability to induce migration of leukocytes.⁵ The CXC family is defined by four conserved cysteine residues; the first two are separated by one nonconserved residue (hence the CXC designation). This group can be further subdivided on the basis of the presence of an N-terminal tripeptide motif glutamate-leucine-arginine (ELR) adjacent to the CXC motif. All ELR⁺-CXC chemokines act through CXC chemokine receptor type 1 or type 2 (CXCR1 and CXCR2), which are rhodopsin-like seven-transmembrane G-protein–coupled receptors. CXCR1 exhibits a high affinity for CXCL8 (IL-8) but a 10- to 100-fold lower affinity for CXCL6 (GCP-2), CXCL7 (NAP-2), or CXCL1 (GRO-α/MGSA-α).⁶,⁷ Rat species possess two homologous receptors, CXCR1 and CXCR2, and four ELR⁺-
CXC chemokines (CXCL1, CXCL2, CXCL5, and CXCL7). However, they lack an equivalent for CXCL8, the key human ELR\(^+\)-CXC chemokine.

Besides their “classical” function of providing migrational signals for leukocytes in adults, ELR\(^+\)-CXC chemokines promote angiogenesis, cell proliferation, and survival during development.\(^6\)–\(^10\) CXCL8 stimulated cell migration, proliferation, and differentiation in the developing intestine and central nervous system\(^11,12\) and enhanced endothelial cell survival and proliferation and the production of matrix metalloproteinases for matrix reconstruction and angiogenesis.\(^10\)

Because they are expressed by UB cells, we hypothesized that ELR\(^+\)-CXC chemokines may also function in metanephric differentiation, cell proliferation, survival, angiogenesis, or migration. In this study, we demonstrate that these chemokines are expressed in the metanephrors and that they promote survival, angiogenesis, and cell migration. Furthermore, we report that Wilms’ tumors express CXCR2, suggesting that tumorigenesis may depend in part on these factors.

RESULTS

Expression of CXC Chemokines and Their Receptors in Rat Embryonic Kidney

Because metanephric inductive factors have heretofore been identified primarily through tedious protein purification methods, we sought to elucidate the majority of factors through genomic analysis. We applied Affymetrix Microarray Gene Chip technology (Affymetrix, Santa Clara, CA) to our cell lines RUB1 and, for comparative purposes, RIMM-18. From these studies, we found that besides known UB cell markers (e.g., Claudin 3, Claudin 9, Bmp3, Bmp7, c-Met, Cytokeratins 18 and 19 [data not shown]), RUB1 cells expressed members of the ELR\(^+\) group of chemokines, namely, Cxcl1, Cxcl2, and Cxcl5. Expression of these chemokines in uninduced RIMM-18 cells was negligible in comparison with RUB1 cells, and both lines failed to express ELR\(^+\) chemokines Cxcl4 and Cxcl10 (Figure 1A).

Because immortalized RUB1 cell expression profiles may differ from those of primary tissues, we also evaluated expression in freshly isolated 13-d postcoitus (dpc) UB or MM, 16- or 19-dpc metanephric, and adult kidneys from rats by reverse transcriptase–PCR (RT-PCR) for all members of the ELR\(^+\)-CXC subfamily and their common receptors Cxcr1 and Cxcr2. RNA from RUB1 or RIMM-18 cells was included to confirm microarray findings. At 13 dpc, both MM and UB expressed Cxcl2, Cxcl5, and Cxcl7 (Figure 1B), although levels by semiquantitative RT-PCR were higher for all of these in the MM. Whereas the RIMM-18 cell line expressed Cxcl7 like its MM progenitor, the RUB1 cell line differed significantly from the UB progenitor cells in that Cxcl1 (and not Cxcl7) was highly expressed, suggesting an adaptive change with culturing. The expression of Cxcl7 in MM was confirmed by Western blotting (Figure 1C), and in situ hybridization demonstrated chemokine expression in both the UB and cortical MM (Figure 1E, purple staining). These studies demonstrate that ELR\(^+\)-CXC family members are expressed at the earliest stages of metanephric development in both inductor UB and nephron progenitor MM, and they persist throughout renal development.

To assess renal cell competence to respond to ELR\(^+\) chemokines, we evaluated kidney tissues at various stages of development by immunoblotting for CXCR1 and 2. By RT-PCR and immunoblotting, both receptors were present in rat kidney from 13 dpc through birth but not in adult kidney (Figure 1, C and D). The data indicate that both UB and MM progenitor populations express Cxcr1 and 2, and this is supported by the observation that CXCR2 is detectable by immunoblotting and RT-PCR (data not shown) in the RUB1 and RIMM-18 cell lines. Immunohistochemistry with anti-CXCR2 antibody revealed a prominent staining in UB, cortical mesenchyme, and newly formed epithelia such as S-shaped bodies (Figure 1F). These findings indicate that this receptor is widely expressed in metanephric progenitors during development and suggest that both UB and MM progenitors are capable of responding to ELR\(^+\) chemokines.

CXC Chemokines Do Not Induce Differentiation in MM

Because CXC chemokines have been implicated in a number of biologic processes relevant to embryogenesis (progenitor cell differentiation, cell survival and proliferation, cell migration/invasion, and angiogenesis), we assessed their effects on these various processes. For this, we used an explant culture system of isolated uninduced MM from 13-dpc rat kidneys or intact metanephiroi of the same age. Culture conditions require the addition of fibroblast growth factor 2 (FGF2) and TGF-\(\alpha\), known survival factors for MM\(^13,14\) and endothelial cells.\(^15,16\)

To minimize their inductive and angiogenic effects, we sought to limit levels of these factors in the culture medium. We found that 30 ng/ml FGF2 and 20 ng/ml TGF-\(\alpha\) maintained the survival of explanted MM but did not induce morphologic changes in the explants. However, such concentrations slightly induced expression of Cxcl1 and Cxcl2 (Figure 2, second column versus first), so tissues were unavoidably exposed to these chemokines as a result of endogenous production when cultivated \textit{ex vivo}. Conversely, Cxcl7 was downregulated in cultured MM under these conditions. In efforts to control for endogenous exposure, experiments included both uncultured and explant cultured MM and the specific CXCR2 inhibitor SB225002\(^17\) in some studies.

For testing whether ELR\(^+\)-CXC chemokines function in tubular development, explanted MM was treated with CXCL7 and examined on subsequent days for markers of tubular differentiation, sfbp2, Lim1, and E-cadherin. Whereas treatment of control cultures of MM with conditioned medium from RUB1 cells induced expression of these markers (Figure 3A) and tubule formation, as previously demonstrated,3 CXCL7 treatment failed to induce expression of markers (Figure 3) or tubular morphogenesis in MM even after 12 d in culture, sug-
suggesting that ELR+CXC chemokines do not function in tubule development.

**ELR+CXC Chemokines Function in Cell Survival**

ELR+CXC chemokines can enhance cell survival and proliferation of some cultured cell types (e.g., human umbilical vein endothelial cells, tumor cell lines). Using a 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell viability test, we assessed the growth effects of CXCL2 or CXCL7 on RIMM-18 cells or primary MM explants. However, we observed no growth advantage for cells that were incubated for 9 d with a wide range of chemokine concentrations (50 to 2000 ng/ml; data not shown). Inhibition of CXCR2 activity using the selective antagonistic compound SB225002 caused massive cell death of both primary MM and RIMM-18 cells. Administration of SB225002 to 13-dpc metanephroi impaired renal morphogenesis in a concentration-dependent manner. Both UB branching and MM tubulogenesis were greatly affected (Figure 4), but administration of SB225002 to embryonic kidney at later stages of development (14- or 16-dpc kidneys) was significantly less inhibitory as shown by staining with TO-PRO-1 reagent (Figure 5). This staining for nonviable cells

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**Figure 1.** Expression of CXCR1 and CXCR2 receptors and their ligands in rat metanephric progenitors, metanephroi, and renal cell lines. (A) Affymetrix data for GeneChip probes D11445 (rat gene Gro-α/CXCL1), U45965 (MIP-2/Gro-β/CXCL2), U90448 (LIX/CXCL5), rc_AI169104 (PF4/CXCL4), and U17035 (IP10/mob-1/CXCL10). RNA was purified from the RIMM-18 or the RUB1 cells and processed as described in the Concise Methods section. (B and D) Semiquantitative reverse transcriptase–PCR (RT-PCR). (C) Western blot analysis of protein extracts from 16-d postcoitus (dpc), 19-dpc, newborn, adult kidney, and 13-dpc metanephric mesenchyme (MM) with CXCR1 (sc-988, Santa Cruz), CXCR2 (sc-683, Santa Cruz), or CXCL7 antibody (AF1116; R&D Systems, Minneapolis, MN). (E) In situ hybridization of 16-dpc rat kidney probed with a CXCL7 antisense riboprobe. (F) Immunohistochemical staining of 16-dpc rat kidney for CXCR2. Bar = 100 μm.
revealed that undifferentiated MM in the cortical nephrogenic zone is more sensitive to SB225002 treatment. For 13-dpc kidney, it involved almost the entire metanephros except the central area where the UB is located (Figure 5B). At 16 dpc, only the cortical nephrogenic zone of kidney was affected (Figure 5F). At higher magnification, nephron formation was observed despite SB225002 treatment, although there are many fewer condensates (as visualized by WT1 staining) in the periphery of the metanephros (Figure 6, A and B). UB branching was also inhibited in the periphery (Figure 6, D and F versus C and E), but staining with TO-PRO-1 does not indicate that cells in the UB are dying in greater numbers than in untreated cultures (Figures 5, E and F, and 6, G and H), suggesting that it is a secondary effect as a result of loss of the MM.

ELR⁺/H11545-CXC Chemokines Function in Angiogenesis

Another established property of ELR⁺/H11001-CXC chemokines is stimulation of angiogenesis. This process implies proteolytic degradation of the basement membrane, proliferation of the two major cell types that populate the microvasculature (endothelial and vascular smooth muscle cells [i.e., the pericyte and mesangial cell]), migration of endothelial cells, and tube formation and fusion with other blood vessels.

To evaluate directly the response of MM to chemokine signaling and determine genomic profiles after stimulation, we screened Affymetrix GeneChips for expression of genes that were upregulated in explanted MM by treatment with CXCL7. Such analyses revealed genes that were associated with survival and with angiogenesis, including endothelial and mesangial markers as well as metalloprotease Mmp9 (Table 1, Figure 7A), which functions in invasive growth, kidney organogenesis, and vasculogenesis.18,19 The upregulated genes included markers of early vascular development: Vegf, Fgfr1, Fgfr2, Tgfβ1 and its receptor TgfβRII, and urokinase-type Plasminogen Activator. Results were confirmed by semiquantitative RT-PCR as were other markers of mesangial and endothelial cells, platelet-endothelial cell adhesion molecule (Pecam-1), Thy-1, and Megas (Figure 7, B and C). Cultivation of MM for 1 d in the presence of FGF2 and TGF-α resulted in upregulation of these markers in comparison with uncultivated MM, but addition of CXCL7 induced much higher levels of expression. In addition, elevation of PECAM-1 in CXCL7-treated MM explants was confirmed by Western analysis (Figure 7D).

For assessment of a possible role of chemokine signaling in
cell migration/invasion, isolated MM and RIMM-18 cells were treated with CXCL7 and evaluated using Matrigel invasion chambers. In these experiments, addition of CXCL7 significantly increased the invasiveness of both MM and RIMM-18 cells through a layer of Matrigel (Figure 8). Typically, we observed a 15 to 35% increase in invasion of MM (Figure 8B). To confirm that this activity was dependent on signaling through CXCR2, we treated cultures with SB225002. In these studies, this CXCR2-specific inhibitor dramatically decreased MM and RIMM-18 cell invasiveness through a Matrigel layer without affecting cell migration through a control membrane, suggesting that the inhibitor was applied at nontoxic levels. These studies demonstrate that CXCL7 can elicit an angiogenic response in embryonic kidney by upregulation of \textit{Mmp9} and endothelial and mesangial markers and further suggest that renal progenitors may use CXCR2 signaling for tissue invasion.

Chemokine Signaling in Renal Tumors

Chemokine signaling has been reported to play a role in the pathogenesis of a variety of tumors, and CXCR1 or 2 specifically has been implicated in the growth or metastasis of melanomas and lung and colon tumor cells. Because Wilms’ tumors originate from MM and caricature metanephric development, we speculated that they would express CXCR and respond to the CXCR2 inhibitor SB225002. To evaluate the potential cell-selective toxicity of SB225002, we compared its effect on RUB1 and RIMM-18 cells as well as on two Wilms’ tumor cell lines, SK-NEP-1 and WiT49, and three renal cell carcinoma lines, CRL, Caki-1, and Caki-2. All analyzed cell lines showed expression of the CXCR2 receptor, but SB225002 induced apoptosis only in blastemal/mesenchymal cell lines (RIMM-18, SK-NEP-1, and WiT49 lines) and not RUB1 cells, as demonstrated by caspase-3 activity or poly(ADP-ribose) polymerase (PARP) cleavage (Figure 9). Administration of a high (2 µg/ml) concentration of CXCL7 to SB225002-treated cells to activate CXCR1 did not rescue the cells from apoptosis (data not shown).

**Figure 5.** The toxic effect of SB225002 depends on the stage of kidney development. Metanephroi at ages 13 (A and B), 14 (C and D), or 16 dpc (E and F) were cultured for 20 h and then treated (B, D, and F) or not (A, C, and E) with 1.1 µM SB225002. After 24 h of incubation, explants were stained with 2% TO-PRO-1 reagent (Molecular Probes) for 30 min at 37°C. Magnification, ×40.

**Figure 6.** Inhibition of CXCR2 signaling with SB225002 primarily affects the cortical nephrogenic zone of cultured metanephroi. (B, D, F, and H) One day of treatment with 1.1 µM SB225002. (A, C, E, and G) Untreated explants. (A and B) Immunostaining with WT1 antibody (arrows show WT1-positive structures: condensed mesenchyme and nephron epithelia). (C and D) DBA staining of ureteric bud (UB). (E and F) Skeletonized images of DBA-stained UB. (G and H) TO-PRO-1 staining of nonviable cells (brackets denote peripheral blastemal area). Magnification, ×100.
shown). We also evaluated primary tumors for CXCR2 and found expression in all Wilms’ tumors but not in renal cell carcinomas (Figure 10). These data support a role for CXCR2 in maintaining survival of normal mesenchymal progenitors and Wilms’ tumors but not epithelial progenitors or renal carcinomas.

DISCUSSION

Chemokines have been described as chemotactic agents and activators of leukocytes during physiologic and inflammatory processes; however, more recent investigations have revealed a nonhematopoietic role for these factors, particularly for the ELR\(^+\)-CXC chemotactic cytokines, which promote mitosis, modulate apoptosis, enhance cell survival, and stimulate angiogenesis. Accordingly, their receptors, CXCR1 and CXCR2, originally shown to be expressed in leukocytes, now have been reported on nonhematopoietic cells (e.g., keratinocytes, neurons, neuroendocrine, and oligodendroglial cells).

More recently, receptors have been described in a variety of embryonic populations. CXCR2 is widely distributed in human tissues including brain (undifferentiated neurons), heart (myocardocytes), lung (bronchial epithelial cells), liver (hepatocytes), and kidney (early glomeruli and collecting duct). In mouse embryos, CXCR2 expression was detected in brain, cardiovascular system, and condensing cartilage. The temporal pattern of receptor expression and the wide embryonic tissue distribution argue for a role in organogenesis and the kidney in particular, yet the role(s) of ELR\(^+\)-CXC chemokines and their receptors in development remains unclear.

This report describes the first evidence of possible functions for ELR\(^+\)-CXC chemokines in metanephric development. We found by RT-PCR and Western blot analysis that in rat embryos, both CXCR2 and CXCR1 are expressed in MM and the UB. Western blot analysis of both receptors showed one major band for each, which corresponds in size to reported forms. Receptor expression was slightly downregulated at birth (more obvious for CXCR1) and subsequently lost from adult kidneys. These findings are consistent with CXCR1 expression patterns obtained by screening rat kidney at different pre- and postnatal stages using microarray.

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For ELR\(^+\)-CXC chemokines, bind CXCR2 with high affinity. Whereas CXCL8 exhibits a high affinity for CXCR1 as well, CXCL1, CXCL6, and CXCL7 show approximately a 100-fold lower affinity for this receptor. In agreement with this, two concentration optima were observed in the effects of CXCL1 and CXCL7 on neutrophil chemotaxis. Because rodents lack a CXCL8 equivalent, signaling through CXCR1 depends on other CXC chemokines. In these studies, we show that CXCL2 and CXCL7 are expressed in rat metanephric tissues, including 13-dpc UB and MM. Adult human renal cells can express CXCL8, CXCL1, or CXCL7 with proinflammatory stimuli. Here, we demonstrate that, under conditions of normal growth and development, ELR\(^+\)-CXC chemokines are expressed and presumably function in organogenesis. Because MM is avascular up to 14 dpc, CXC chemokines that are synthesized directly by renal progenitors may be the only renal source of CXCR ligands. Of course, we cannot rule out the possibility that some noted effects resulted from changes in the microenvironment as a result of culturing. For example,
CXCL1 expression increased in cultured rudiments, whereas CXCL7 expression was downregulated. The differences, though, were quantitative and not qualitative in nature and therefore probably reflect either normal compensatory responses or possible feedback regulatory relationships among family members. The significance of such changes, however, is unclear, because ligand substitutions may not have a significant impact on CXCR signaling.

The angiogenic properties of ELR⁺/H11001-CXC chemokines are well documented, so it is reasonable to hypothesize such a role in the metanephros. Although the origin of endothelial elements in the metanephros remains controversial and may arise from more than one tissue source, there is sufficient evidence to indicate that they originate at least in part from the endogenous blastemal population.³⁷ A comparison of gene expression profiles of uninduced MM that were treated with CXCL7 revealed a group of genes related to angiogenesis. We detected elevated levels of endothelial and mesangial markers (Pecam-1 and Megsin) and other angiogenesis-related genes (Mmp9 and Vegf). We did not apply hypoxic conditions for MM cultivation, which are normal for embryonic development and a stimulus to angiogenesis. In cultured MM, the hypoxia-induced angiogenic stimulator Vegf was significantly downregulated relative to primary tissue isolates (data not shown), so it is possible that exposure of explants to hypoxia could further enhance the effect of CXCL7 on these markers, including Vegf.

In these studies, the induction of some markers (e.g., Pecam-1, Vaa, Pdgfr) was greatest using very high concentrations of CXCL7 (2.0 μg/ml). This may reflect a contribution of CXCR1, which has a lower affinity for CXCL7 and, unlike CXCR2, is rapidly re-expressed upon internalization.³⁹ Lower concentrations of CXCL7 still induced these markers but may be less effective as a result of the upregulation of Mmp9, which inactivates the chemokine.⁴⁰ Conversely, MMP9 may contribute to angiogenesis by degrading extracellular matrix and facilitating the sprouting of growing blood vessels.¹⁹ The findings on metalloprotease upregulation obtained by Affymetrix and RT-PCR assays were supported by migration/invasion studies. We detected a measurable basal rate of invasion by cells from the MM, presumably as a result of the presence of FGF2 and TGF-α, which are necessary to sustain these cells and can induce metalloprotease activity.⁴¹,⁴² Cultured MM had a six-fold greater expression level for Mmp9 than freshly isolated MM. In addition, Matrigel itself contains multiple growth factors, which may also facilitate invasiveness. Despite this high basal level, we observed a significant increase in invasion of MM upon stimulation with CXCL7. The CXCR2 antagonist SB225002 inhibited both basal and CXCL7-stimulated invasion of MM at nontoxic concentrations, supporting the role of the receptor in cell motility/invasion. RIMM-18 cells gave similar results, except cells were less dependent on added growth factors, presumably as a result of E1A immortalization.

Genomic profiling of CXCL7-treated MM also revealed a
A group of apoptosis- and growth-related genes. CXCL7-mediated downregulation of antiproliferative (Gadd45, Btg2), apoptosis-inducing (Pdcd8), and tumor suppressor (Brca2) genes and upregulation of Fgfr1 and Fgfr2 genes argue for a prosurvival role of CXCL7. In fact, targeted loss of both Fgfr genes in MM results in renal agenesis, so their expression is essential to MM survival. However, proapoptotic genes (Anxvin V and Caspase 3) were also upregulated with CXCL7 administration. This may explain why an MTT test showed no growth advantage for CXCL7-treated MM. More probable, any proapoptotic effects may be offset by the observed upregulation of CXCL1 and CXCL2 in control MM cultures, which may be sufficient to maintain the survival of explants independent of CXCL7 in culture medium, but this will require further investigation.

It seems, however, that CXCR2 is critical for survival of undifferentiated MM, because SB225002 caused its selective death. MM cells from metanephroi or established mesenchymal lines, including two Wilms’ tumors, were sensitive to SB225002-induced apoptosis, unlike renal epithelial (UB) cells, suggesting that blastemal cells require signaling through CXCR2. It is known that phosphatidylinositol-3 kinase/Akt and extracellular signal–regulated kinase prosurvival signaling cascades can be triggered via CXCR2. Furthermore, neu-
eralization of CXCR2 signaling was shown to modulate anti- and prosurvival proteins in endothelial cells and negatively affect their survival.46 However, identification of the specific mechanism(s) responsible for caspase 3–dependent, SB225002-initiated apoptosis in MM cells is to be the subject of future study. Impairment of UB branching after SB225002 administration seems to be a secondary event, because UB cells (both primary and from the RUB1 cell line) did not show signs of apoptosis or death. In all probability, the loss of branching may be due to the loss of MM-secreted branch-inducing factors, such as glial cell line–derived neurotrophic factor (GDNF).47 Participation of CXCR2 in the survival of blastemal cells in kidney may be critical, because levels of CXCL7 that could activate the CXCR1 receptor did not rescue the cells from SB225002-induced apoptosis. In this regard, it is possible that the induction of angiogenic markers by CXCL7 is indirect and due instead to enhanced survival of angiogenic progenitors as a part of blastemal cell population. For nonblastemal cells, other signaling pathways may contribute to survivability, even though they express CXCR2.

Although ELR⁺-CXC chemokines have been implicated in the control of cell differentiation,48,49 we did not detect morphologic changes that are characteristic of mesenchymal-to-epithelial conversion of MM or even CXCL7-induced expression of specific epithelial markers. Although this may simply reflect the inadequacies of cell culture conditions, it may also be attributable to the cooperative nature of inductive signaling as we have demonstrated.3 Therefore, CXCL7 may function in tubulogenesis in combination with other factors.

The results obtained for CXCR2-deficient mice confirm the angiogenic role of the receptor. Decreased vascular density as well as marked reduction of tumor growth and its metastatic role of the receptor. Decreased vascular density as the control of cell differentiation,48,49 we did not detect mor-

CONCISE METHODS

Cell Cultures

RUB1 and RIMM-18 cell lines were established from rat UB and undifferentiated MM, respectively, and were characterized previously and grown as described.44 Wilms’ tumor line WT149 was a gift of Dr. Herman Yeger (Hospital for Sick Children, Toronto, Canada). Other tumor lines were obtained from the ATCC (Rockville, MD).

Isolation and Cultivation of Rat Embryonic Kidneys and MM

Embryonic kidneys were excised from F344 rat embryos. MM were enzymatically separated from embryonic day 13.5 UB. Metanephroi or MM were cultured on polycarbonate filters (Whatman-Nucleapore, Florham Park, NJ) coated with type IV collagen (BD Bioscience, Bedford, MA) as described previously.1

Affymetrix GeneChip Analysis

RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA (5 μg) from duplicate samples was converted into cDNA and purified by phenol/chloroform extraction. cDNA labeling, hybridization to U34A GeneChips, and scanning were performed according to Affymetrix instructions, and data were analyzed by GeneChip software.

Semiquantitative RT-PCR Analysis

RT reactions and PCR amplifications were performed using total RNA (0.2 or 1 μg) as described previously.54 Primer sequences, annealing temperatures, and numbers of cycles are shown in Table 2. No RT controls were included to eliminate the possibility of DNA contamination. All PCR products were sequenced to confirm identities.

Western Blot Analysis

Protein lysates were obtained from rat kidneys at various stages of development (16 dpc, 19 dpc, newborn, or adult) or from isolated 13-dpc MM (20 to 30) and analyzed by Western blotting as described previously.54

Immunocytostaining

MM or kidney explants cultivated on polycarbonate filters were fixed in methanol, washed, preblocked with 10% sheep serum (Sigma-Aldrich, St. Louis, MO), and stained for WT1 (rabbit polyclonal, 1:50; Santa Cruz Biotechnology, Santa Cruz, CA) or PCR-1 (mouse monoclonal, 1:50; Chemicon Int., Temecula, CA), using Alexa488-conjugated secondary antibodies (1:100; Molecular Probes, Eugene, OR) in 1% sheep serum overnight at 4°C. Filters were washed, stained if needed with 20 μg/ml Dolichos biflorus agglutinin (Sigma), and mounted using a ProLong Antifade Kit (Molecular Probes).

In Situ Hybridization and Immunohistochemistry

Frozen sections (20 μm) of 4% paraformaldehyde-fixed, 30% sucrose permeabilized, and OCT-embedded 16-dpc rat kidneys were probed with a 200-bp digoxigenin-labeled riboprobe according to Tuttle et al.55 using a chromogenic alkaline phosphatase substrate BM Purple (Roche, Palo Alto, CA). For immunohistochemistry, similarly prepared frozen sections were probed with a rabbit polyclonal antibody for CXCR2 (sc-683, Santa Cruz Biotechnology) or nonimmune rabbit IgG at a 1:100 dilution. Staining was visualized using a Vectastain ABC
kit (Vector Laboratories, Burlingame, CA), and sections were lightly counterstained with hematoxylin.

Measurement of Caspase-3 Activity Using a Fluorometric Substrate

Caspase-3 activity in cells was measured using 20 μM caspase-3 fluorometric substrate Ac-DEVD-amc (Upstate Biotech, Lake Placid, NY) and 1 μM caspase-3 inhibitor IV Ac-VEID-CHO (for negative control; Calbiochem, San Diego, CA), according to manufacturers’ instructions. Fluorescence was measured at excitation 380 nm and emission 460 nm using a Luminescence Spectrometer (Perkin-Elmer LS50B, Waltham, MA). Protein contents were quantified with a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL), and fluorescence was normalized to protein content.

Invasion Assay

Invasiveness of RIMM-18 or MM cells was determined using BD Biocoat Matrigel Invasion Chambers (BD Biosciences) according to the manufacturer’s recommendations. RIMM-18 cells (3 × 104 cells/well) or MM (1 MM/well) were added to the invasion chambers in 500 μl of medium with FGF2 (10 ng/ml); 700 μl of the same medium was added to the culture wells. CXCL7 (200 ng/ml; Peprotech, Rocky Hill, NJ) was added to the culture wells; inhibitors, when applied, were added to both the invasion chambers and the culture wells. After 20 h of incubation, non-invading cells were removed with cotton swabs, and membranes were fixed in 100% methanol and stained with Giemsa solution.

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DISCLOSURES

None.

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