Autocrine CSF-1 and CSF-1 Receptor Coexpression Promotes Renal Cell Carcinoma Growth

Julia Menke1,2, Jörg Kriegsmann2, Carl Christoph Schimanski2, Melvin M. Schwartz3, Andreas Schwarting2, and Vicki R. Kelley1

Abstract

Renal cell carcinoma is increasing in incidence but the molecular mechanisms regulating its growth remain elusive. Coexpression of the monocytic growth factor colony-stimulating factor (CSF)-1 and its receptor CSF-1R on renal tubular epithelial cells (TEC) will promote proliferation and antiapoptosis during regeneration of renal tubules. Here, we show that a CSF-1–dependent autocrine pathway is also responsible for the growth of renal cell carcinoma (RCC). CSF-1 and CSF-1R were coexpressed in RCCs and TECs proximally adjacent to RCCs. CSF-1 engagement of CSF-1R promoted RCC survival and proliferation and reduced apoptosis, in support of the likelihood that CSF-1R effector signals mediate RCC growth. In vivo CSF-1R blockade using a CSF-1R tyrosine kinase inhibitor decreased RCC proliferation and macrophage infiltration in a manner associated with a dramatic reduction in tumor mass. Further mechanistic investigations linked CSF-1 and epidermal growth factor signaling in RCCs. Taken together, our results suggest that budding RCC stimulates the proximal adjacent microenvironment in the kidney to release mediators of CSF-1, CSF-1R, and epidermal growth factor expression in RCCs. Furthermore, our findings imply that targeting CSF-1/CSF-1R signaling may be therapeutically effective in RCCs. Cancer Res; 72(1); 187–200. ©2011 AACR.

Introduction

Renal cell carcinoma has been steadily on the rise for several decades. Renal clear cell carcinoma (RCC) is derived from proximal tubule epithelial cells and is by far the most common (70%–80%) form of kidney carcinomas (1, 2). However, despite the increased incidence of RCCs, the molecular mechanisms that regulate the growth of this tumor remain elusive (3).

Colony-stimulating factor (CSF)-1 and its receptor (CSF-1R) are instrumental during the progression of epithelial tumors of the female reproductive tract and prostate (4–7). For example, CSF-1 and CSF-1R are coexpressed in more than 50% of mammary tumors and elevated circulating CSF-1 levels are an indicator of early metastatic relapse in patients with breast cancer (8, 9). In addition, CSF-1 expression in primary breast carcinoma and RCC correlates with infiltration of inflammatory cells and, in turn, poor prognosis of this tumor (10). In fact, CSF-1 generated by mammary tumors and RCC recruit and activate large numbers of tumor-associated Mo (TAMS) that release trophic cytokines and other growth factors, thereby enhances mammary and renal carcinoma growth and facilitate tumor metastases (10–14). Moreover, an autocrine loop in breast cancer cell lines expressing both CSF-1 and the CSF-1R may contribute to tumor invasion and metastasis (4, 9, 15). Furthermore, recent findings indicate that CSF-1R is expressed on tubular epithelial cells (TEC) in RCC; however the relevance to the development and progression of this tumor was not elucidated (5, 16). Thus, signaling through the CSF-1R may promote the progression of renal epithelial cell tumors.

Inflammation is meant to set the stage for repair. We recently uncovered a CSF-1–dependent mechanism of renal tubular repair (17). After transient renal ischemia, CSF-1 and CSF-1R are coexpressed on TECs, including those in the proximal tubule, in mice and humans. CSF-1 engaging with the CSF-1R induces TECs to proliferate and inhibits further apoptosis leading to the replenishment of injured TECs. CSF-1 is integral in the healing process as CSF-1 injected into mice after ischemia/reperfusion (I/R) hastens tubular healing whereas blocking the CSF-1R prevents renal tubular regeneration (17, 18). However, CSF-1 was originally identified as the principle Mo developmental molecule that stimulates survival, differentiation, proliferation, and activation of Mo (19, 20), and CSF-1 has a sole receptor, the c-fms tyrosine kinase proto-oncogene that is expressed on cells of the monocyte lineage (Mo, dendritic cells; ref. 21). As Mo are implicated in the repair of numerous tissues (22), we probed for the contribution of a CSF-1–dependent Mo along with autocrine TEC renal repair after I/R. We determined that CSF-1–mediated tubular repair is dependent on TEC autocrine Mo-independent and
Mø-dependent mechanisms following transient injury (17). Thus, signaling via the CSF-1R on TECs and Mø is intended to protect the kidney by mediating tubular repair.

To probe for the mechanisms that promote RCC growth, we hypothesized that: (i) RCCs coexpress CSF-1 and the CSF-1R and (2) the CSF-1–mediated autocrine feedback loop, intended to promote tubular repair in normal kidneys is "hijacked" by the RCC and instead triggers tumor cell proliferation and inhibits tumor cell apoptosis, thereby promoting tumor growth.

Materials and Methods

Renal biopsy specimens

Renal carcinoma tissues (discarded tissues) with a confirmed pathologic diagnosis were provided by the Department of Pathology, Rush University Medical Center, Chicago, IL, and the Department of Pathology, Johannes-Gutenberg University, Mainz, Germany. RCC samples were analyzed using the morphologic classification of the carcinomas according to World Health Organization (WHO) specifications to evaluate clinical outcome.

CSF-1R and CSF-1 expression

**Immunofluorescence.** In vitro, we cultured RCC (786-0 and Caki), HK2, and MCF-7 lines according to the manufacturer’s instructions (Cell Line Services). Cells (1 × 10^5 per well) were stimulated with TPA [12-O-tetradecanoylphorbol 13-acetate] for 48 hours. We fixed cells with methanol for 5 minutes on coverslips and incubated them with rabbit-anti-mouse CSF-1R Ab or rabbit IgG (Santa Cruz) for 1 hour at room temperature. We detected CSF-1R by incubating cells with anti-rabbit Cy3 Ab (Vector) for 30 minutes. We mounted the coverslips with Vectashield with 4',6-diamidino-2-phenylindole (DAPI; Vector) and analyzed them using a Nikon Eclipse E1000 upright fluorescence microscope.

**Immunohistochemistry.** Paraffin sections: We determined CSF-1 (CSF-1-Ab; N-16; Santa Cruz), phospho-M-CSFR (rabbit anti-human phospho-M-CSFR Tyr 723 Ab; 49C10; Cell signalling), and CSF-1R (CSF-1R-Ab; Santa Cruz) as previously described (23). We determined the number of positive cells in 10 randomly selected high-power fields and evaluated the corresponding areas within serial sections for the correlation analysis.

**CSF-1/EGF ELISA.** To quantify the levels of CSF-1 in supernatants, we evaluated samples using an ELISA for each as previously described (23). We analyzed epidermal growth factor (EGF) in supernatants using an ELISA (R&D Systems) according to the manufacturer’s instructions.

**Human cell lines**

The following cell lines were used and cultured as previously described: immortalized RCC lines (786-0 and Caki), immortalized proximal TEC line (HK2; ref. 24); promyelocytic leukemia cell line (HL60; ref. 25); and immortalized T-cell line (JURKAT). Carcinoma cells were purchased from the Cell Line Services.

TECs stimulated with RCC and Mø supernatant

To determine CSF-1R and CSF-1 expression on TECs induced by mediators released by RCC and Mø, we stimulated RCC cell lines and Mø with TNF-α (6 ng/mL), TNF-α/LPS (6 and 12 ng/mL) and phorbol-12-myristate-13-acetate (PMA) for 24 hours. Then, the supernatant was removed and fresh media were added. After an additional 24 hours, the supernatant (undiluted and diluted: 1:1, 1:4, and 1:8 with media) was added to cultured TECs (HK2). After 48 hours, the CSF-1 in the cultured TEC supernatant was evaluated (ELISA assay) and CSF-1 and CSF-1R transcript expression on cultured TEC was evaluated by quantitative real-time PCR (qRT-PCR). We measured CSF-1 concentrations in the supernatant of RCCs and Mø before coincubation with TECs to determine the baseline expression of CSF-1 in these cells. CSF-1 expression in TECs that were not stimulated served as controls.

**CSF-1R and CSF-1 transcript expression**

We analyzed the CSF-1R and CSF-1 expression in human cell lines using real-time, 2-step, quantitative PCR as previously described (qRT-PCR; ref. 26). The mRNA levels were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). We used the following PCR primers: GAPDH: sense, 5’-CCC TCA ACG ACC ACT TTG TCA-3’; antisense, 5’-TTT CTC TTG TGC TCT TGC TTG-3’; CSF-1R: sense, 5’-TGA AGA CTA GGA CAA GGA-3’; antisense, 5’-CCA TTG GTC AAC AGC ACG AGC AGC-3’. CSF-1: sense, 5’-GCC ATG CAA CCA CAA CAA CAA-3’; antisense, 5’-AAG CAG TAA CTG AGC AAC AAC GGG-3’.

**CSF-1R RNA interference**

For transient inhibition of CSF-1R mRNA production, 786-0 and HK2 cell lines were transfected with a commercially available pool of 4 target-specific 20 to 25 nucleotide-long siRNAs designed to "knock down" CSF-1R expression and with a control siRNA (Santa Cruz Biotechnology, Inc.). For stable inhibition of CSF-1R expression, 786-0 and HK2 cell lines were transfected using a short hairpin RNA (shRNA) plasmid [pool of 4 target-specific lentiviral vector plasmids each encoding 19–25 nucleotide-long (plus hairpin) shRNAs are designed to target the CSF-1R knockdown] or control shRNA plasmid (Santa Cruz Biotechnology). Each plasmid contains a puromycin resistance gene to select cells stably expressing shRNA. These transfections were conducted with shRNA Plasmid Transfection reagent according to the manufacturer’s instructions (Santa Cruz Biotechnology).

**Preparation of von Hippel–Lindau in 786-0^+ cell line**

Using a retroviral gene transfer approach, 293T cells were transfected with pBABe-puro-VHL DNA and a control DNA as previously described (27). 786-0 cells expressing the von Hippel–Lindau (VHL) gene were selected with puromycin (1 μg/mL).

**Proliferation**

**Immunohistochemistry.** We stained paraffin sections using a primary Ab against rabbit anti-human/mouse Ki67 (SP6c Lab Vision) to identify proliferating TECs.
**MTT assay.** We cultured RCC [786-0 and Caki-1 (Caki), Caki-2], human TEC (HK2), and human breast carcinoma (MCF-7) lines in 96-well plates (5 × 10³ cells per well) for 12 hours and stimulated these cells for 72 hours with human recombinant CSF-1 or EGF (Sigma-Aldrich) at various concentrations (5, 20, 40, and 80 ng/mL). To verify specificity by blocking the CSF-1R, we incubated cells with CSF-1 in combination with CSF-1R Ab (25 ng/mL; Santa Cruz) or CSF-1R tyrosine kinase inhibitor [5-(3-methoxy-4-[(4-methoxybenzyl) oxy]benzyl)-pyrimidine-2-4-diamine; Calbiochem] or with EGF and anti-EGFR Ab for 72 hours. We used control antibodies for CSF-1R Ab (rabbit IgG) and EGFR Ab (mouse IgG2B) in all experiments. Furthermore, we stimulated cultured RCC, HK2, and MCF-7 lines with varying concentrations of TNF-α (3, 6, 15, and 30 ng/mL), lipopolysaccharide (LPS; 6, 12, 25, and 50 ng/mL; Sigma-Aldrich) in combination with CSF-1R Ab (25 ng/mL; Santa Cruz) or anti-EGFR Ab. We analyzed proliferation using the MTT colorimetric assay (Roche) according to the manufacturer’s instructions.

**Apoptosis**

We cultured RCC, HK2, and MCF-7 lines in 6-well (apoptosis assay) or 96-well (proliferation assay) plates (5 × 10⁴ per well) for 12 hours and stimulated these cells with varying concentrations of human recombinant CSF-1 (5, 20, 39, and 78 ng/mL) in combination with TNF-α (3, 6, 15, and 30 ng/mL), lipopolysaccharide (LPS; 6, 12, 25, and 50 ng/mL; Sigma-Aldrich) and TNF-α for 72 hours. We assessed apoptosis by flow cytometry using an Annexin-V-FITC-PI kit (BD Bioscience) according to the manufacturer’s instructions.

**Mice**

We purchased athymic nu/nu BALB/c mice from The Jackson Laboratory. Mice were housed at Johannes-Gutenberg University. The use of mice in this study was reviewed and approved by the Standing Committee on Animals at the University of Mainz.

**Implanting TECs under the renal capsule**

We implanted RCC (786-0) or HK2 cells under the renal capsule of athymic nu/nu BALB/c mice at 3 months of age as previously described (27).

**Treatment with CSF-1R tyrosine kinase inhibitor**

We injected mice (intraperitoneally) every 24 hours with the CSF-1R tyrosine kinase inhibitor (Calbiochem; 25 mg/kg/body weight) beginning at 1.0 day after surgery and ending at 21 days after surgery. The CSF-1R inhibitor is a cell-permeable diaminopyrimidine compound that acts as a potent, selective, and ATP-competitive inhibitor of c-fms kinase activity (IC₅₀ = 30 nmol/L) with minimal inhibition toward a panel of 26 other kinases (IC₅₀ > 5 µmol/L). It is shown to selectively inhibit c-fms–mediated cellular functions in vitro as well as CSF-1–dependent tumor growth in vivo (28).

**Statistical analysis**

The data represent the mean ± SEM and were prepared using GraphPad Prism version 4.0. We used the nonparametric Mann–Whitney U test to evaluate P values. For correlation analysis, we used the Spearman correlation calculation.

**Results**

**CSF-1R and CSF-1 are expressed in RCC but not papillary carcinoma**

CSF-1 and the CSF-1R are coexpressed on TECs following a transient ischemic injury and via an autocrine/paracrine mechanism–mediated renal tubular repair (17). Moreover, the CSF-1R is expressed on epithelial cells of carcinomas including breast and prostate (6, 29). Thus, we hypothesized that CSF-1 and the CSF-1R are coexpressed on RCC. To test this hypothesis, we probed for CSF-1 and CSF-1R protein expression in human kidney carcinomas and RCC and papillary carcinoma specimens in comparison with normal kidney by immunostaining. We detected robust CSF-1R expression in RCCs as compared with normal kidney that lacked CSF-1R expression (Fig. 1A). Because the CSF-1R is internalized following activation, CSF-1R is expressed on the membrane and in the cytoplasm (30, 31). To determine whether CSF-1R expression is restricted to the RCCs, we examined the TECs adjacent and distant to the RCCs. We detected ubiquitous expression of CSF-1R on TECs adjacent to RCCs, whereas CSF-1R expression was limited to far fewer TECs distant to RCCs (Fig. 1A). In fact, there was a CSF-1R expression gradient emanating from the RCCs and declining in proportion to the distance from the tumor. To determine whether CSF-1 is expressed along with CSF-1R in RCCs, we stained for the presence of CSF-1 in RCCs compared with normal kidney. We detected CSF-1 expression along with CSF-1R expression in RCCs. Moreover, the CSF-1 expression was most pronounced in the TECs contiguous to the tumor, diminished with distance from RCCs, and was not detectable in the areas most distant to the tumor. Furthermore, we detected a correlation in the expression of CSF-1R and CSF-1 in RCCs and the TECs adjacent, but not distant, to RCC (Fig. 1B). In contrast, we rarely detected CSF-1R and CSF-1 expression in papillary carcinoma (Fig. 1A). Taken together, CSF-1 and CSF-1R are ubiquitously coexpressed in RCCs and TECs closest to the RCCs and declines with distance from the tumor.

**CSF-1R and CSF-1 expression in RCCs and TECs adjacent to RCCs correlates with infiltrating Mo but not T cells**

Increased numbers of Mo in human breast cancer is an index of poor prognosis (32, 33). Moreover, mammary tumor metastasis is attenuated in mice with reduced numbers of Mo (34). To determine whether enhanced expression of CSF-1R and CSF-1 on TEC in RCC fosters Mo and T-cell–incited inflammation, we probed for Mo and T cells in RCC and the areas adjacent and distant to the tumor. We detected a greater magnitude of Mo in RCCs than in normal kidneys (Fig. 2A). Moreover, Mo were more abundant in the RCC than in the area adjacent and distant to RCC. In fact, the magnitude of CSF-1R and CSF-1 expression correlated with CD68⁺ Mo in RCCs and TECs adjacent, but not distant, to RCCs (Fig. 2B). Of note, we did not detect an increase in CD3⁺ T cells in RCCs, nor in TECs adjacent and distant to RCCs (Fig. 2A). And CSF-1R and CSF-1 expression did not correlate with the number of T cells in the...
RCCs and the adjacent TECs (Fig. 2B) and we did not detect an increase in T cells in papillary carcinoma (Fig. 2A). Taken together, our findings suggest that the magnitude of CSF-1R and CSF-1 is an index of the extent of Mø, but not T cells, in RCCs and the adjacent TECs.

Coexpression of CSF-1 and CSF-1R in RCCs correlates with TEC proliferation

CSF-1 engaging with CSF-1R on the same or adjacent TEC induces proliferation, thereby replenishing necrotic and apoptotic TEC following transient ischemia (17). To determine whether the CSF-1R and CSF-1 coexpression of TECs in RCCs has an impact on TEC proliferation resulting in an increase in RCCs, we assessed CSF-1, CSF-1R, and proliferation in TECs in sequential kidney sections from patients with RCCs. The magnitude of CSF-1 and CSF-1R expression was greatest in proliferating (Ki67⁺) TEC in RCCs and TECs adjacent to RCCs (Fig. 3). Moreover, we determined that CSF-1R⁺ TECs expressed the tyrosine-phosphorylated CSF-1R, indicating that the CSF-1R is signaling on the TEC (Fig. 3). Taken together, this suggests that coexpression of CSF-1 and CSF-1R on RCCs and TECs adjacent to RCCs promotes
proliferation and is thereby responsible for promoting the expansion of this tumor.

We next investigated whether coexpression of CSF-1R, phospho-CSF-1R, and CSF-1 on proliferating TECs correlated with clinical disease. The criteria for clinical disease included tumor morphology (stage and grade/dedifferentiation) and patient survival. We evaluated 22 patients with RCC (average age of 64 years, 60% male and 40% female). We found that CSF-1R and CSF-1 were associated with enhanced Ki67 expression and with accelerated tumor progression (tumor stage and grade and reduced survival of the patients; Supplementary Fig. S1). Moreover, as the phospho-CSF-1R expression was similarly associated with tumor progression, this indicates that the CSF-1R was functional (Supplementary Fig. S1). This suggests that coexpression of CSF-1 and CSF-1R on RCCs hastens the progression of RCCs by promoting proliferation and differentiation.

Figure 2. CSF-1R and CSF-1 expression on RCCs and TECs that are adjacent to RCCs correlates with infiltrating Mø, but not CD3⁺ T cells.

A, we evaluated the presence of CD68⁺ and CD3⁺ leukocytes in formalin-fixed sections by immunostaining. We confirmed staining specificity using isotype control Abs. Representative photomicrographs are shown (magnification 20×, enlargement 40×). B, correlations of CSF-1R and CSF-1 expression with CD68⁺ and CD3⁺ leukocytes in RCCs and the TECs in areas adjacent and distant to RCCs. Values are the mean ± SEM. hpf, high-power field.
Mediators predominantly released from RCC increase CSF-1R and CSF-1 expression on adjacent TEC

To test the hypothesis that increased expression of CSF-1R and CSF-1 on TECs adjacent to RCCs is induced by mediators released by RCC and/or TAMS, we conducted a series of in vitro experiments. Incubating the TEC cell line (HK2) with RCC (786-0) supernatant led to a concentration (1:1-1:8)-dependent increase in CSF-1 protein (Supplementary Fig. S2) and transcripts (Fig. 4, bottom) as compared with TECs not incubated with RCC supernatant. Following RCC stimulation (TNF-α), the concentration-dependent increase in CSF-1 expression was even higher than in unstimulated RCCs (Fig. 4, top). Baseline expression of RCCs before coincubation with TEC is displayed as a dotted line.

By comparison, incubating TECs with increasing concentrations of Mø (HL60) supernatant modestly increased CSF-1 expression on TECs (Fig. 4, top; Supplementary Fig. S2). Furthermore, we detected a similar pattern of CSF-1R transcript expression on TECs following incubation with supernatant generated by RCCs and Mø (Fig. 4, bottom). This suggests that mediators predominantly released by RCCs induce CSF-1 and CSF-1R expression on TECs adjacent to RCC.

**CSF-1 engaging with CSF-1R on RCC promotes survival/proliferation, thereby fostering RCC growth**

We hypothesized that CSF-1 engaging with CSF-1R on TEC promotes survival/proliferation in RCCs. To test this...
hypothesis, we first established that human RCC cell lines, 786-0 (Fig. 5) and Caki-1 (Caki; wild-type VHL) and Caki-2 (devoid of VHL), express CSF-1R and CSF-1 transcripts and protein. We detected more robust CSF-1R transcript and protein (immunofluorescence) expression on TECs in RCC lines than on a human proximal tubule line (HK2) and a breast carcinoma line (MCF-7). And CSF-1R transcript expression increased when RCC line was stimulated with PMA (Fig. 5; Supplementary Fig. S3). In parallel with CSF-1R expression, we detected an upregulation of CSF-1R transcripts and protein expression following stimulation with TNF-α expressed in TAMS (35) along with LPS, a conventional stimulant, in cultured RCC cell lines. CSF-1 upregulation by TNF-α/LPS was even more robust than in HK2 and MCF-7 cells (Fig. 5B; Supplementary Fig. S5). Thus, the CSF-1R and CSF-1 expression is not dependent on the VHL mutation.

Because inactivating mutations in the VHL tumor suppressor gene are associated with RCC (36), we investigated whether CSF-1 and CSF-1R expression in RCCs is dependent on VHL. We did not detect a difference in CSF-1R and CSF-1 expression comparing the Caki (wild-type VHL) with Caki-2 (VHL-deficient) cell line (data not shown) or the 786-0 RCC line with and without VHL expression (786-0-VHL + /786-0-VHL −). Furthermore, CSF-1 stimulated a similar increase in proliferation/survival in the Caki/Caki-2 and 786-0-VHL + /786-0-VHL − cell lines (Supplementary Fig. S5). Thus, the CSF-1R and CSF-1 expression is not dependent on the VHL mutation.

CSF-1 engaging with CSF-1R on RCC suppresses apoptosis, thereby increasing RCC growth

CSF-1–dependent dampened apoptosis contributes to renal tubular repair, following transient ischemic injury (17). Therefore, we hypothesized that CSF-1 mediates an increase in RCC mass via CSF-1–dependent suppression of apoptosis in RCC. To test this hypothesis, we blocked the CSF-1R along with TNF-α/LPS stimulation to increase CSF-1 expression and evaluated survival/proliferation (as above). We detected a decrease in RCC mass following blockade of the CSF-1R (by CSF-1R Ab, transient and stable silencing by siRNA and shRNA), enhanced in RCC with prior TNF-α/LPS stimulation (Fig. 6B and C, data not shown for siRNA and shRNA). Thus, RCC generates sufficient CSF-1 to trigger CSF-1R signaling on RCCs resulting in enhanced RCC survival/proliferation, thereby increasing the tumor growth.

Blocking CSF-1R signaling in vitro inhibits RCC growth

We hypothesized that blocking the CSF-1R suppresses RCC growth. To test this hypothesis, we evaluated the impact of a CSF-1R tyrosine kinase inhibitor on human RCC in vitro. We stimulated an RCC line with increasing concentrations of CSF-1 and added the CSF-1R tyrosine kinase inhibitor. The CSF-1R
tyrosine kinase inhibitor suppressed CSF-1-dependent proliferation and survival (data not shown) of the RCC line. Thus, the CSF-1R tyrosine kinase inhibitor effectively dampens renal tumor cell growth.

To determine whether blocking the CSF-1R on RCC inhibits RCC growth in vivo, we constructed an RCC model in BALB/c mice. We implanted human RCC (786-0) under the renal capsule in athymic nu/nu BALB/c mice, as these cells will not be rejected. To block the CSF-1R, we compared mice injected with a CSF-1R tyrosine kinase inhibitor (28, 37) and PBS for 21 days (Fig. 7). We implanted human TECs (HK2) under the renal capsule to serve as a negative control. We detected a dramatic decrease in local tumor growth following CSF-1R tyrosine kinase inhibitor compared with PBS treatment (Fig. 7). However, provision of the CSF-1R tyrosine kinase inhibitor did not reduce the RCC mass to baseline (level of implanted normal human TECs Fig. 7). Thus, CSF-1R tyrosine kinase blockade suppresses human RCC growth. To determine whether the reduced tumor cell expansion is a result of reduced RCC proliferation, we evaluated the number of proliferating (Ki67+\(^{+}\)) RCC. We detected fewer proliferating RCCs in the CSF-1R tyrosine kinase inhibitor–treated mice than in PBS-injected mice (Fig. 7). This further supports the concept that RCCs are stimulated to proliferate via a CSF-1/CSF-1R autocrine/paracrine feedback loop. Mø have been implicated in the promotion of human carcinomas (15). Thus, we evaluated the number of kidney-infiltrating Mø within the implant site. The number of Mø (CD68\(^{+}\)) decreased in CSF-1R tyrosine kinase–treated mice comparable with the mice implanted with HK2 cells (Fig. 7). In fact, the magnitude of Mø in CSF-1R tyrosine kinase–inhibited mice was reduced to the level of those implanted with nonmalignant HK2 cells. Taken together, blocking the CSF-1R effectively suppresses the growth of RCCs.

**EGF mediates CSF-1–dependent RCC increased proliferation and suppressed apoptosis**

Breast carcinoma cells generate CSF-1 that, in turn, stimulates Mø to express EGF and EGF stimulates breast carcinoma cells to express CSF-1 (4, 15). Thus, we hypothesized that EGF stimulates RCCs to express CSF-1, thereby promoting proliferation. To test this hypothesis, we incubated RCCs with EGF and measured CSF-1 in the supernatant and cell mass. We detected a progressive increase in the level of CSF-1 released in the supernatant (Fig. 8A), accompanied by increased proliferation of RCCs in response to EGF (Fig. 8B). As expected, blocking the EGF receptor (EGFR) suppressed EGF-mediated CSF-1–stimulated TEC proliferation. Moreover, inhibiting EGFR along with CSF-1R additively suppressed RCC proliferation. In conclusion, the interaction of CSF-1 and EGF enhances the expansion of RCC by promoting RCC proliferation (Supplementary Fig. S6).

To determine whether EGF-stimulated CSF-1 suppresses apoptosis of RCC cells, we stimulated RCCs with TNF-α/LPS. Adding EGF dampened apoptosis of RCCs.
apoptosis is specific for EGF as blocking with anti-EGFR Ab restored apoptosis and replacing the EGFR Ab with a control (mouse IgG2b) did not restore apoptosis. To determine whether EGF stimulates CSF-1 and thereby promotes proliferation and dampens apoptosis in RCCs, we repeated the studies in Fig. 8B and C and blocked the CSF-1R. Adding anti-CSF-1R Ab to the EGF-stimulated TECs decreased proliferation (Fig. 8B) and restored apoptosis (Fig. 8C), thereby supporting the concept that EGF mediates proliferation and apoptosis via a CSF-1R-dependent mechanism. Thus, EGF induces CSF-1–dependent increased RCC proliferation and reduced apoptosis.

**CSF-1 and CSF-1R expression on TEC adjacent to RCC is mediated, in part, by EGF**

CSF-1 and the CSF-1R are upregulated on TECs adjacent, not distant, by mediators released primarily from RCCs (Fig. 4). Therefore, we tested the hypothesis that EGF is released by RCCs and is responsible for inducing CSF-1 and CSF-1R on TECs adjacent to RCCs. Stimulating RCC and TEC lines with EGF increases CSF-1 expression (Fig. 8A). Thus, we hypothesized that the TECs adjacent to the RCCs are stimulated by RCCs and TAMS to release EGF. Incubating the TEC cell line (HK2) with supernatant of TNF–α/LPS–stimulated RCC (786-0) or Mø (HL60) led to a concentration-dependent increase in EGF expression (Fig. 8D; Supplementary Fig. S2, bottom) as compared with TECs that were not incubated with RCC or Mø supernatant. This suggests that RCCs and TAMS release mediators that induce EGF expression on TECs adjacent to RCCs that, in turn, upregulate CSF-1/Csf-1R expression and lead to an increase in TEC proliferation and a reduction in TEC apoptosis.

**Discussion**

We now report the novel finding that autocrine CSF-1–dependent RCC mechanisms are central to the growth of RCCs. While recent findings indicate that the CSF-1–R is expressed on RCCs (5, 16), our study clarifies the CSF-1–mediated
mechanisms central to promoting RCCs. In summary, CSF-1R and CSF-1 are abundantly coexpressed in RCC and in intra-renal TECs adjacent, but not distant, to RCCs. The engagement of CSF-1 with its sole cognate receptor, CSF-1R, promotes RCC survival and proliferation and reduces apoptosis. This suggested that signaling via the CSF-1R promotes RCC growth. Moreover, blocking the CSF-1R with a CSF-1R tyrosine kinase inhibitor in human RCC xenografts in mice decreases the survival and proliferation of the RCC and the infiltration of Mø, thereby leading to a dramatic reduction in tumor mass. Furthermore, EGF induces CSF-1 and the CSF-1R on RCCs, promoting tumor cell proliferation and dampening tumor cell apoptosis. RCC has an impact on the kidney microenvironment adjacent to the tumor, as it releases mediators that induce CSF-1, CSF-1R, and EGF expression on these TECs. Taken together, we suggest that targeting the CSF-1R is a potential therapeutic approach for human RCCs.

Our findings highlight the CSF-1–dependent autocrine mechanism that fosters RCC growth. A wealth of evidence has detailed the involvement of CSF-1–bearing TAMS in tumor progression and metastasis (11, 38). The paracrine interactions between tumor cells and TAMS facilitate the spread of a tumor in the host by promoting tumor cell migration, invasion, and metastasis (4, 15). As CSF-1 generated by the tumor is essential in regulating TAMS, CSF-1– and CSF-1R–expressing Mø are key components in the tumor microenvironment regulating the fate of the host. To identify CSF-1–dependent TAMS-mediated mechanisms central to tumor progression, investigators studied tumors lacking the CSF-1R, thereby eliminating CSF-1–dependent tumor autocrine mechanisms (34, 39). For example, CSF-1 blockade in human colon, devoid of CSF-1R, indicates that CSF-1–dependent Mø-mediated molecular mechanisms regulate tumor growth (40). However, malignant tumors such as breast and ovary coexpress CSF-1 and the CSF-1R (5, 7, 9). Thus, CSF-1 and CSF-1R coexpression on breast and ovarian carcinoma prompted investigations to uncover a CSF-1–dependent autocrine loop that contributes to tumor invasiveness and metastasis.

Figure 7. Blocking CSF-1R signaling in vivo inhibits RCC growth. A, CSF-1 stimulates proliferation (MTT assay) of RCC (786-0) and blocking with CSF-1R tyrosine kinase inhibitor suppresses CSF-1–mediated RCC proliferation, HK2 line served as a positive control. Results are representative of 2 separate experiments; means ± SEM. B, RCC (786-0) and HK2 cells were implanted under the renal capsule of athymic nu/nu BALB/c mice. CSF-1R tyrosine kinase inhibitor was injected beginning 1 day after surgery for 21 days. Mice were sacrificed 22 days following surgery. Growth (mass), proliferation (Ki67), and infiltration of CD68+ leukocytes in RCCs was suppressed in mice treated with the CSF-1R tyrosine kinase inhibitor compared with PBS treated controls. Proliferating cells and CD68+ leukocytes are circled. Representative photomicrographs (T = tumor). Values are means ± SEM; n = 4–5 per group. Hpf, high-power field.
Moreover, heightened proliferation is evident in mammary glands of CSF-1 transgenic mice that result in mammary tumors. Our findings uniquely highlight the pivotal position of a CSF-1–dependent autocrine mechanism in the most common kidney tumor in human RCCs. While a recent report indicates that the CSF-1R is expressed in RCC, using sequential sections from patients with RCC, our findings illustrate CSF-1 and CSF-1R expression.

**Figure 8.** EGF mediates CSF-1–dependent enhanced proliferation and suppressed apoptosis of RCC. 
A, EGF stimulates RCCs to express CSF-1. CSF-1 measured in the supernatant of RCC cells (786-0 and Caki) by ELISA. Results are representative of 3 separate experiments. Values are means ± SEM. B, EGF stimulates proliferation of RCC cells and anti-EGFR Ab or anti-CSF-1R Ab suppresses EGF-dependent proliferation of RCC cells. HK2 cell line served as a positive control. Proliferation assessed by the MTT assay. Values are means ± SEM. Results are representative of 4 separate experiments. C, EGF-mediated CSF-1 expression suppresses RCC apoptosis. RCCs were cultured for 72 hours in the absence of CSF-1. Cells were stimulated with concentrations of TNF-α and LPS. EGF stimulation dampens apoptosis and blocking the CSF-1R with anti-CSF-1R Ab or blocking the EGFR with anti-EGFR Ab increases TNF-α/LPS–induced TEC apoptosis. Means ± SEM; n = 6 per group. D, RCCs and Mø release mediators that stimulate TEC to express EGF. RCCs (786-0) and Mø were stimulated with TNF-α. Supernatants were added to the cultured TEC cell line (HK2) undiluted (neat), diluted at 1:1, 1:4, and 1:8 with media from stimulated RCCs (786-0) or Mø (HL60). EGF expression was measured by ELISA. Note, comparable results stimulating RCC and Mø with TNF-α/LPS and TPA (data not shown).
coexpression on proliferating TECs and establish that the CSF-1R is phosphorylated. Also, we detect enhanced tumor cell proliferation and dampened tumor cell apoptosis, proportional to CSF-1 expression in RCC lines, thereby indicating that RCC growth is mediated by CSF-1. Moreover, an increase in TAMS in the RCC and kidney adjacent to RCC suggests that, as in other cancers (38), CSF-1R–expressing Mø may contribute to the progression of RCCs. Taken together, CSF-1–dependent mechanisms are central to RCCs.

Mediators released by the RCC maybe instrumental in altering the TECs adjacent to the RCCs. We determined that CSF-1 and the CSF-1R are upregulated on the TECs adjacent, but not distant, to RCCs. As TAMS are more abundant in the kidney adjacent to RCCs, this suggests that mediators generated by the RCCs and/or TAMS induce CSF-1 and CSF-1R on the TECs closest to the tumor. We determined that the RCCs, and to a far lesser extent Mø, release mediators, including EGF, that induce CSF-1 and the CSF-1R on TECs. There are several possible consequences of upregulating CSF-1 and the CSF-1R coexpression on TECs adjacent to RCCs. It is possible that RCC injures the TECs closest to the tumor and as we previously reported, CSF-1 and CSF-1R coexpression triggers TEC proliferation and dampens TEC apoptosis, thus replenishing damaged tubules (17). On the other hand, upregulated CSF-1 and the CSF-1R on TECs may be harmful. For example, overexpression of EGFR promotes proliferation and in mutated EGFR leads to oncogenesis (43, 44). Thus, upregulated CSF-1 and CSF-1R coexpression on TECs adjacent to RCCs enhances autocrine TEC proliferation, and along with exposure to additional triggers, may lead to malignant transformation of TECs. Alternatively, our findings indicate that CSF-1, generated by TECs adjacent to RCCs, contributes to Mø recruitment. Thus, it is possible that these Mø are instrumental in promoting tumor growth. Despite the consequence of upregulating these molecules in TECs adjacent to RCCs, it is clear that RCC releases mediators that trigger CSF-1 and CSF-1R expression in the kidney microenvironment.

Is the CSF-1R a therapeutic target for human RCC? We now report that blocking the CSF-1R on RCCs dramatically suppresses RCC growth. CSF-1R tyrosine kinase inhibitor, known to block the CSF-1R in vivo (28, 37) delivered into a human RCC xenograft mouse (human RCC cells implanted under the kidney capsule of athymic nude mice), dramatically reduced RCC mass. Our findings are consistent with other therapeutic approaches (antisense, interfering RNA, antibodies) that target the CSF-1R in other forms of cancer (colon, mammary) and lead to tumor suppression (40, 45, 46). However, our findings are in contrast to the failure of a mouse anti-CSF-1R Ab to inhibit tumor growth in subcutaneous mesothelioma and Lewis lung carcinoma models (47). Recalling that Mø, and some malignant tumors, express the CSF-1R, blocking the CSF-1R may suppress tumor expansion via CSF-1–dependent autocrine and paracrine mechanisms. While these tumors (mesothelioma and lung) are similar to RCC as they originate from cells of epithelial origin, CSF-1R expression on these tumors was not explored. And despite decreasing TAMS using this anti-CSF-1R Ab, the CSF-1–dependent mechanisms that may regulate these particular tumors have not been detailed. Thus, CSF-1R blockade may be a promising therapeutic approach for some, but not all, malignant tumors. It is worth noting that sunitinib, a multitargeted receptor tyrosine kinase inhibitor, that targets the CSF-1R along with many other receptor tyrosine kinases, is a first-line therapy for advanced RCCs (1, 48, 49). Taken together, our findings clearly indicate that targeting the CSF-1R is a potential therapeutic for human RCCs. Detailing the relative contribution of autocrine CSF-1–dependent and the unexplored potential of Mø-mediated paracrine mechanisms will be central to tailoring therapeutic strategies to halt human RCCs.

CSF-1 and EGF expression and functions are closely linked in the kidney during inflammation. Following acute renal injury, heparin-binding EGF is upregulated on TECs and promotes TEC proliferation, findings that parallel CSF-1 expression and function (50–52). Moreover, an increase in CSF-1R and EGFR expression correlates with poor prognosis of human tumors (43, 44). And in mammary tumors, blocking EGFR and CSF-1R inhibits carcinoma invasiveness (53). Finally, CSF-1 and EGF are components of a positive feedback loop. EGF generated by Mø induces CSF-1 expression in breast carcinoma cells, and in turn, CSF-1 generated by breast carcinoma cells induces EGF expression in Mø (15). Thus, we hypothesized that EGF induces CSF-1 in RCCs. We determined that EGF stimulates CSF-1 expression in RCC lines and promotes RCC cell proliferation and dampens RCC cell apoptosis. As blocking EGFR and CSF-1R reverses these EGF-incited findings, we suggest that EGF induces CSF-1 in RCC. In addition, we determined that RCCs, and to a lesser degree Mø, release mediators that induce EGF expression and, in turn, CSF-1/CSF-1R expression on TECs and thereby modify the kidney microenvironment.

In conclusion, we have identified CSF-1–dependent autocrine mechanisms that are instrumental in fostering RCC growth. Taken together, an autocrine CSF-1–dependent mechanism, intended to promote tubular repair following renal injury, has been subverted and now drives tumor expansion. Our findings suggest that CSF-1R is a potential therapeutic target for human RCCs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Michaela Blanfeld for excellent technical support.

Grant Support

This work was supported by NIH grants DK-36149 (VRK), Alliance for Lupus Research (V.R. Kelley), and the Deutsche Forschungsgemeinschaft (ME-3194/1-1, to J. Menke).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 14, 2011; revised September 6, 2011; accepted October 13, 2011; published OnlineFirst November 3, 2011.
CSF-1–Dependent Growth of Renal Carcinoma

References


Cancer Res; 72(1) January 1, 2012

www.aacrjournals.org

199

Published OnlineFirst November 3, 2011; DOI: 10.1158/0008-5472.CAN-11-1232

Downloaded from cancerres.aacrjournals.org on April 13, 2017. © 2012 American Association for Cancer Research.


Autocrine CSF-1 and CSF-1 Receptor Coexpression Promotes Renal Cell Carcinoma Growth

Julia Menke, Jörg Kriegsmann, Carl Christoph Schimanski, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-11-1232

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2011/11/03/0008-5472.CAN-11-1232.DC1

Cited articles
This article cites 49 articles, 24 of which you can access for free at:
http://cancerres.aacrjournals.org/content/72/1/187.full.html#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
/content/72/1/187.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.