CXCR3-B Can Mediate Growth-inhibitory Signals in Human Renal Cancer Cells by Down-regulating the Expression of Heme Oxygenase-1*

The chemokine receptor CXCR3 may play a critical role in the growth and metastasis of tumor cells, including renal tumors. It has been shown that CXCR3 has two splice variants with completely opposite functions; CXCR3-A promotes cell proliferation, whereas CXCR3-B inhibits cell growth. We recently demonstrated that the expression of growth-promoting CXCR3-A is up-regulated, and the growth-inhibitory CXCR3-B is markedly down-regulated in human renal cancer tissues; and the overexpression of CXCR3-B in renal cancer cells can significantly inhibit cell proliferation. However, the growth-inhibitory signal(s) through CXCR3-B are not well characterized. Here, we investigated the effector molecule(s) involved in CXCR3-B-mediated signaling events. We found that the overexpression of CXCR3-B in human renal cancer cells (Caki-1) promoted cellular apoptosis as observed by FACS analysis through Annexin-V staining. To examine whether the overexpression of CXCR3-B could alter the expression of any apoptosis-related genes in renal cancer cells, we performed a protein array. We found that CXCR3-B overexpression significantly down-regulated the expression of antiapoptotic heme oxygenase-1 (HO-1). By utilizing a HO-1 promoter-luciferase plasmid, we showed that CXCR3-B-mediated down-regulation of HO-1 was controlled at the transcriptional level as observed by luciferase assay. We also demonstrated that the inhibition of HO-1 expression using siRNA promoted apoptosis of renal cancer cells. Finally, we observed that human renal cancer tissues expressing low amounts of CXCR3-B significantly overexpress HO-1 at both mRNA and protein level. Together, we suggest that the overexpression of CXCR3-B may prevent the growth of renal tumors through the inhibition of antiapoptotic HO-1.

Chemokines are small cytokine-like secreted proteins that have chemotactic properties and are well established to function in the recruitment of leukocytes into inflamed tissues (1, 2). The biologic effects of chemokines are mediated through specific G protein-coupled receptors (3). Besides their functions in the immune system, chemokines and chemokine receptors also play a critical role in tumor initiation, progression, and metastasis (4–6). Recent studies clearly suggest that in several malignancies, tumor cells express different chemokines and their receptors that are associated with more aggressive disease and poorer prognosis (7–12).

The chemokine receptor CXCR3 is classically expressed on T cells (3, 13); however, recent reports have shown that it may also be expressed in different types of tumor cells, including renal tumors (7, 9–11, 14, 15). CXCR3 can interact with its specific ligands, CXCL9, CXCL10, and CXCL11 (13). It has been reported that CXCR3 is alternatively spliced in different human tissues to produce two known variants, CXCR3-A and CXCR3-B (7, 13, 16–18). Some tissues, such as heart, kidney, liver, skeletal muscle, and human airway epithelial cells, express both splice variants, whereas other tissues, such as placenta and human mesangial cells, express only CXCR3-A, and human microvascular endothelial cells selectively express CXCR3-B. Each of the CXCR3 splice variants mediates completely different intracellular signals and has distinct functions (13, 16, 17). CXCR3-A promotes chemotaxis and cell proliferation, whereas CXCR3-B signals for growth inhibition (7, 16, 18, 19).

We have recently demonstrated that the expression of the growth-promoting CXCR3-A is markedly high in human renal cancer cells; in contrast, the expression of the growth-inhibitory CXCR3-B is significantly low in these cells (18). We suggest that in the absence/low presence of CXCR3-B, renal cancer cells undergo increased proliferation and migration through CXCR3-A-mediated signaling events. In support of this finding, we observed that the overexpression of CXCR3-B indeed inhibited renal cancer cell proliferation (18). Thus, it is likely that signaling through CXCR3-B in renal cancer cells may inhibit cell growth.

It is now established that the postreceptor signals via CXCR3-B result in an increase in intracellular cyclic AMP levels, whereas CXCR3-A mediates calcium influx within the cell (16, 19, 20). Although coupling of the G proteins to chemokine receptors is important for their biological activities, CXCR3-A and CXCR3-B may mediate their functions through different G protein coupling (7, 16, 19). Petrai et al. (21) showed that the growth-inhibitory signal through CXCR3-B is mediated by the p38 mitogen-activated protein kinase (MAPK) pathway. However, the effector molecules for CXCR3-B-mediated signaling events in terms of tumor growth/inhibition are not characterized.

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Heme oxygenase-1 (HO-1)\(^2\) is a rate-limiting enzyme in heme degradation, and it converts heme to biliverdin, carbon monoxide (CO), and iron (22–24). It plays an important protective role in the tissues by reducing oxidative injury, attenuating the inflammatory response, inhibiting cell apoptosis, and regulating cell proliferation (22, 25, 26). Although it is a cytoprotective enzyme, it has been reported that HO-1 activation may play a significant role in carcinogenesis and can influence the growth and metastasis of tumors (27–29). HO-1 is often highly up-regulated in tumor tissues, and the overexpressed HO-1 may promote tumor growth. It has been suggested that the inhibition of HO-1 expression can be a potential therapeutic approach to sensitize tumors to radiation and chemotherapy (30–32).

In this study, we show that HO-1 is significantly expressed in human renal cancer tissues and that the overexpression of CXCR3-B in human renal cancer cells can markedly down-regulate HO-1 expression at both transcriptional and protein levels. We also demonstrate that the overexpression of CXCR3-B and the inhibition of HO-1 can lead to augmented apoptosis of these cells. These observations suggest that CXCR3-B may mediate growth-inhibitory signal(s) in renal cancer cells through down-regulation of HO-1 expression.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The small interfering RNA (siRNA) for HO-1 and its control were purchased from Qiagen. The transfection of siRNA was performed using Lipofectamine 2000 (Invitrogen). Cobalt protoporphyrin (CoPP) was obtained from Frontier Scientific.

**Cell Line**—The human renal cancer cell line Caki-1 was obtained from American Type Culture Collection. The cells were grown in McCoy's medium supplemented with 10% fetal bovine serum (Invitrogen).

**Tissue Samples**—Tissue samples of human renal cell cancer (RCC) were obtained from surgical specimens of patients who underwent surgery at the University Hospital (Würzburg, Germany). The protocol to obtain tissue samples was approved by the review board of the hospital. Tumor tissues were graded (stages I through IV) according to Robson staging system. Normal renal tissues were obtained from normal tissue specimens, and the normalcy of these tissues was confirmed by histology.

**Plasmids**—CXCR3-B overexpression plasmid (pcDNA3-CXCR3-B) was a gift from P. Romagnani (University of Florence, Florence, Italy) (21). The human HO-1 promoter-luciferase construct was a gift from J. Alam (Alton Ochsner Medical Foundation, New Orleans, LA). The plasmid pHO4luc was constructed by cloning the promoter fragment from the human HO-1 gene (bp ~4067 to 70 relative to transcription start site) into the luciferase reporter gene vector pSKluc.

**Flow Cytometry**—To measure intracellular CXCR3 expression, the cells were fixed/permeabilized in Cytofix/Cytoperm solution (BD Biosciences) for 20 min at 4 °C and washed with Perm/Wash solution (BD Biosciences). The fixed cells were then incubated with FITC-conjugated antibody to total CXCR3 (R & D Systems) or matched IgG isotype for 30 min at 4 °C. The stained cells were analyzed in a FACSCalibur (Becton Dickinson).

**RNA Isolation and Real-time PCR**—Total RNA was prepared using the RNEasy isolation kit (Qiagen), and cDNA was synthesized using cloned avian myeloblastosis virus first-strand synthesis kit (Invitrogen). To analyze mRNA expression, we performed real-time PCR using the Assay-on-Demand Gene Expression product (TaqMan, Mammalian Gene Collection probes) according to the manufacturer’s instructions (Applied Biosystems). As an internal control, 18 S mRNA was amplified and analyzed under identical conditions. Gene-specific primer-probe sets for human CXCR3-B/HO-1/18 S were obtained from Applied Biosystems. The Ct value (the cycle number at which emitted fluorescence exceeded an automatically determined threshold) for the gene of interest was corrected by the Ct value for 18 S and expressed as ΔCt. The fold change of mRNA amount was calculated as follows: (fold changes) = 2^\(X\) (where \(X = ΔCt\) for control group – ΔCt for experimental group).

**Protein Array**—Protein array was performed using apoptosis protein array kit from R & D Systems, according to the manufacturer’s protocol.

**Transfection and Luciferase Assay**—Caki-1 (2.5 × 10^5 cells) were transfected with the CXCR3-B overexpression plasmid or the HO-1 promoter-luciferase plasmid using the Effectene transfection reagent (Qiagen), according to the manufacturer’s protocol. The total amount of transfected plasmid DNA was normalized using a control empty expression vector. Transfection efficiency was determined by co-transfection of the β-galactosidase gene under control of cytomegalovirus immediate-early promoter and by measurement of β-galactosidase activity. For luciferase assay, cells were harvested 48 h after transfection, and luciferase activity was measured using a standard assay kit (Promega) in a luminometer.

**Apoptosis Assay**—The cellular apoptosis was measured by Annexin-V and propidium iodide staining using an FITC Annexin-V Apoptosis Detection kit (BD Pharmingen) according to the manufacturer’s protocol. Following staining, the cells were analyzed by FACS analysis.

**Western Blot Analysis**—Protein samples were run on SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (NEN Life Sciences Products). The membranes were incubated with anti-HO-1 (R & D Systems) or anti-β-actin (Santa Cruz Biotechnology) and subsequently incubated with peroxidase-linked secondary antibody. The reactive bands were detected by chemiluminescence (Pierce).

**Immunohistochemistry**—Immunohistochemistry was performed on frozen sections of human RCC tissues and normal renal tissues. Briefly, acetone-fixed sections were incubated first with anti-human HO-1 (Abcam) and second with a species-specific horseradish peroxidase-conjugated secondary antibody. Specimens were washed thoroughly between incubations, developed in 3,3’-diaminobenzidine (BioGenex), and counterstained in Meyer’s Hemalaun using standard techniques.

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\(^2\) The abbreviations used are: HO-1, heme oxygenase-1; CoPP, cobalt protoporphyrin; FACS, fluorescence-activated cell sorter; RCC, renal cell cancer; ROS, reactive oxygen species.
**Statistical Analysis**—Statistical evaluation for data analysis was determined by Student’s t test. Differences of $p < 0.05$ were considered statistically significant.

**RESULTS**

**Overexpression of CXCR3-B Promotes Apoptosis of Human Renal Cancer Cells**—We first wished to examine whether the overexpression of CXCR3-B in human renal cancer cells could promote apoptosis. The Caki-1 cells were transfected with either the CXCR3-B overexpression plasmid (1.0 μg) or the empty expression vector. Following 48 h of transfection (A) the CXCR3-B mRNA expression was checked by real-time PCR (the lower Ct value reflects overexpression of the gene), and (B) the CXCR3 (A + B) protein expression was measured by FACS analysis. C, the confluence of the cells was evaluated microscopically. D, the apoptotic index of the cells was determined by Annexin-V and propidium iodide staining using an Apoptosis Detection kit as described under “Experimental Procedures.” All results are representative of three independent experiments.

![Graph showing statistical analysis](image)

**Overexpression of CXCR3-B Down-regulates HO-1 Expression in Renal Cancer Cells**—We next questioned whether the overexpression of CXCR3-B could alter the expression of any apoptosis-related protein(s) in renal cancer cells. To this end, we performed a protein array utilizing an apoptosis array kit to examine the change in expression of 35 apoptosis-related genes. The Caki-1 cells were transfected with either the CXCR3-B overexpression plasmid or the empty vector. Following transfection (48 h), the cells were lysed, and the lysates were analyzed for the expression of apoptosis-related proteins as described above. As shown in the Fig. 2, the overexpression of CXCR3-B significantly down-regulated the expression of the antiapoptotic protein HO-1 compared with empty vector-transfected controls. There was also some down-regulation in the expression of HO-2 following CXCR3-B overexpression; however, there was no significant change in the expression of other proteins. It has been reported that both HO-1 and HO-2 are expressed in renal tissues; however, only HO-1, and not HO-2, is significantly elevated in renal cancer (29). This finding suggests that CXCR3-B may mediate a growth-inhibitory apo-
ptotic signal in human renal cancer cells through the down-regulation of antiapoptotic HO-1, which is the inducible isoform of heme oxygenase.

CXCR3-B Overexpression Down-regulates HO-1 Promoter Activity—As our earlier experiment suggested that the overexpression of CXCR3-B can inhibit the expression of HO-1 in renal cancer cells, we next sought to determine whether CXCR3-B could regulate HO-1 promoter activity. The Caki-1 cells were co-transfected with the HO-1 promoter-luciferase construct and either the CXCR3-B overexpression plasmid or the empty vector. The effect of CXCR3-B overexpression on HO-1 promoter activation was assessed by the measurement of luciferase activity in cell lysates. As shown in Fig. 3A, CXCR3-B overexpression significantly inhibited HO-1 promoter activity.

We also confirmed through Western blot analysis that CXCR3-B-mediated down-regulation of HO-1 promoter activity was associated with a decrease in HO-1 at the protein level. As shown in Fig. 3B, transfection with the CXCR3-B overexpression plasmid markedly inhibited HO-1 protein expression in Caki-1 cells, compared with empty vector-transfected controls. Together, these experiments suggest that CXCR3-B can inhibit the expression of antiapoptotic HO-1 by down-regulating its promoter activity.

Down-regulation of HO-1 Is Associated with Increased Apoptosis of Renal Cancer Cells—We next set out to determine whether the down-regulation of HO-1 expression could promote apoptosis in renal cancer cells. To knock down HO-1, the Caki-1 cells were transfected with either HO-1 siRNA or control siRNA. Following transfection with HO-1 siRNA, we performed the apoptosis assay through Annexin V and propidium iodide staining, and the cells were analyzed by FACS analysis as described earlier. As shown in Fig. 4A, we observed that the knockdown of HO-1 in renal cancer cells increased cellular apoptosis compared with control cells; the percentage of early apoptotic cells increased from 0.93% (control cells) to 5.28%. The knockdown of HO-1 was confirmed by Western blot analysis (Fig. 4B).

FIGURE 2. Overexpression of CXCR3-B in renal cancer cells inhibits the expression of antiapoptotic HO-1. Caki-1 cells were transfected with either the CXCR3-B overexpression plasmid (1.0 μg) or the empty expression vector for 48 h. The cells were lysed, and a protein array was performed utilizing an apoptosis protein array kit. The table chart beside the blot indicates the individual proteins tested in this array. Results are representative of three independent experiments.

FIGURE 3. Overexpression of CXCR3-B inhibits HO-1 promoter activity. A, Caki-1 cells were co-transfected with the HO-1 promoter-luciferase construct (1.0 μg) and either different amounts (0.5 and 1.0 μg) of CXCR3-B overexpression plasmid (filled columns) or the empty expression vector (open column). The cells were harvested after 48 h, and the percent decrease in luciferase activity was calculated from luciferase counts of each group of cells compared with that of cells transfected with empty expression vector alone. Results are representative of three independent experiments. Columns, average of triplicate readings of samples; error bars, ± S.D. *, p < 0.01, compared with empty vector-transfected cells. B, Caki-1 cells were transfected with either different amounts (0.5 and 1.0 μg) of CXCR3-B overexpression plasmid or the empty expression vector. Following 48 h of transfection, whole cell lysates were prepared, and Western blot analysis was performed using anti-HO-1 to quantitate HO-1 protein expression. The expression of β-actin in these cells was analyzed by Western blotting using anti-β-actin. Results are representative of three independent experiments.
We also examined the apoptotic index of these renal cancer cells following overexpression of HO-1 through CoPP treatment. As shown in Fig. 5A, we observed that the CoPP treatment significantly inhibited cellular apoptosis compared with vehicle-treated control cells; the percentage of early apoptotic cells decreased from 10.31% (vehicle-treated cells) to 3.47%. The overexpression of HO-1 in CoPP-treated cells was confirmed by Western blot analysis (Fig. 5B). Taken together, the data suggest that HO-1 may mediate a survival signal in human renal cancer cells and the down-regulation of its expression may lead to cellular apoptosis.

**DISCUSSION**

The two splice variants of the chemokine receptor CXCR3 may have completely opposite functions in different cell types; CXCR3-A may promote cell proliferation, whereas CXCR3-B may mediate growth inhibition (7, 16, 19). In the present study, we show that the overexpression of CXCR3-B in human renal cancer cells can mediate a growth-inhibitory effect through the down-regulation of antiapoptotic HO-1 expression.

Reactive oxygen species (ROS), such as superoxide anion radicals and hydrogen peroxide, are potentially harmful by-products of normal cellular metabolism that directly affects cellular function (30, 33). An increased level of ROS may promote significant damage to cell structure and functions (34). HO-1, a member of the heat shock protein family, plays a key role as a sensor and regulator of cellular oxidative stresses (35, 36). HO-1 is induced in tissues during these stress conditions, and it promotes cytoprotection. On the other hand, it has been
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It has been reported that the growth-inhibitory effect of CXCR3-B is mediated through the p38 MAPK signaling pathway (21). Keum et al. (38) have demonstrated that the activation of the p38 MAPK may down-regulate the expression of HO-1 in human cancer cells, and p38 MAPK-mediated inhibition of HO-1 expression is regulated at the transcriptional level. Thus, it is possible that p38 MAPK may act as one of the critical intermediary molecules in CXCR3-B-mediated signaling events for HO-1 inhibition. However, we cannot rule out the role(s) of other intermediary molecules, as the growth-inhibitory signaling event(s) through CXCR3-B is a complex process and needs to be elucidated. It is also possible that CXCR3-B-mediated down-regulation of HO-1 is an indirect response through the inhibition of CXCR3-A-mediated signaling pathway(s).

In summary, we suggest that HO-1 may act as a therapeutic target in human renal cancer. The inhibition of HO-1 may allow the generation of ROS, and the elevated ROS level may facilitate tumor killing. Our observations in this study clearly show that the overexpression of CXCR3-B in renal cancer cells can down-regulate HO-1 expression, and mediate tumor cell apoptosis. Thus, the therapeutic strategies that may induce CXCR3-B-mediated signaling events(s) may prevent/restrict the growth of human renal cell cancer through the inhibition of HO-1 expression.

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