Secreted CXCL1 Is a Potential Mediator and Marker of the Tumor Invasion of Bladder Cancer

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Abstract

Purpose: The purpose of this study was to identify proteins that are potentially involved in the tumor invasion of bladder cancer.

Experimental Design: We searched for the candidate proteins by comparing the profiles of secreted proteins among the poorly invasive human bladder carcinoma cell line RT112 and the highly invasive cell line T24. The proteins isolated from cell culture supernatants were identified by shotgun proteomics. We found that CXCL1 is related to the tumor invasion of bladder cancer cells. We also evaluated whether the amount of the chemokine CXCL1 in the urine would be a potential marker for predicting the existence of invasive bladder tumors.

Results: Higher amount of CXCL1 was secreted from highly invasive bladder carcinoma cell lines and this chemokine modulated the invasive ability of those cells in vitro. It was revealed that CXCL1 regulated the expression of matrix metalloproteinase-13 in vitro and higher expression of CXCL1 was associated with higher pathologic stages in bladder cancer in vivo. We also showed that urinary CXCL1 levels were significantly higher in patients with invasive bladder cancer (pT1-4) than those with noninvasive pTa tumors (P = 0.0028) and normal control (P < 0.0001). Finally, it was shown that CXCL1 was an independent factor for predicting the bladder cancer with invasive phenotype.

Conclusions: Our results suggest that CXCL1 modulates the invasive abilities of bladder cancer cells and this chemokine may be a potential candidate of urinary biomarker for invasive bladder cancer and a possible therapeutic target for preventing tumor invasion.

Bladder cancer is the fourth most common cancer in the United States and it will be responsible for the death of an estimated 13,060 persons in the United States alone during 2006 (1, 2). Bladder cancer is generally classified into superficial (non-muscle invasive) or muscle-invasive cancer based on the natural history of these tumors. Most bladder cancers (~80%) present as superficial tumors, which include Ta (noninvasive) or T1 (lamina propria invasive) tumor. Among these superficial tumors, ~70% recur after transurethral resection and 10% to 20% show progression to become muscle-invasive (T2-4) tumors. T1 tumors are more likely to progress to muscle-invasive disease (25-40%) than Ta tumors (3-4%; ref. 3). Muscle-invasive cancer has a much less favorable prognosis than superficial cancer (4). Due to the unfavorable prognosis of muscle-invasive cancer, there is considerable interest in developing markers that can identify superficial cancers with a high risk of progression. The identification of this type of marker will contribute to the early detection of life-threatening invasive bladder cancer and the improvement of the prognosis of this disease.

Multifocality and frequent recurrence is also another characteristic of bladder cancers (5). Multifocal tumors often present with varying degrees of stage and grade. Most studies have found only monoclonal tumors. The presence of shared genetic changes in all tumors resected from individual patients suggests that these are related lesions that have evolved from a single altered cell clone. However, there are some examples of more than one unrelated monoclonal tumor in the same bladder (oligoclonality), and this is not surprising given the association of transitional cell carcinoma risk with smoking and the pan-urolithal carcinogenic insult associated with this (6). There are two types of bladder cancer with fundamentally...
different genetic alterations and clinical outcome. Whereas noninvasive tumors are genetically stable and characterized by frequent FGFR3 mutations and deletions of chromosome 9q, invasive bladder cancer is characterized by gross chromosomal instability and many genetic alterations. Alterations in both p53 and Rb tumor gene suppressor pathways are well documented in invasive bladder cancer. Additionally, numerous markers, such as cell adhesion molecules, tumor-associated antigens, proliferating antigens, and cell cycle regulatory proteins, have been identified, which correlate to some extent with tumor stage and prognosis (7). However, the power of many of these biomarkers in detecting superficial disease or predicting the clinical outcome of individual tumors is limited, and alternative markers are still in demand.

Secreted proteins determine, control, and coordinate many of the biological processes in organisms, such as cellular growth, differentiation, and tumorigenesis (8). After the completion of the Human Genome Project, many researchers have hypothesized that the best cancer biomarkers will probably be secreted proteins (9). Thus, great interest is currently being focused on the characterization of proteins secreted by isolated tumor cells and neoplastic tissues to identify novel biomarkers and new molecular targets for therapy.

Approximately 20% to 25% of all cellular proteins undergo secretion (10); however, the quantity of each protein secreted is usually very low. Analysis of culture supernatants after incubation of cells seems to be a reasonable way to identify such secreted proteins. However, even minor contamination with protein-rich FCS can easily mask secreted proteins of interest. Recently, we established a new method for analyzing the proteins in cell culture supernatants by using serum-free medium. Using this method, we identified clusterin as a marker for the hypoxia-inducible factor–independent function of von Hippel-Lindau protein and revealed its role in the development of familial pheochromocytoma (11, 12). In these studies, secreted proteins in the serum-free medium were precipitated with trichloroacetic acid and analyzed by two-dimensional PAGE. Then, protein spots of interest were identified by mass spectrometry (MS) analysis. Although two-dimensional PAGE provides information about the position of protein spots, it may not be so effective for detecting scarce secreted proteins (13). In the present study, we improved our method by using multidimensional shotgun proteomics, a powerful proteomics analysis tool with a high efficiency to identify hundreds of proteins in a single run (14, 15). Using this method, we compared the proteome of secreted proteins in the culture supernatant from bladder cancer cell lines with different invasive phenotypes and found that CXCL1 is overexpressed in invasive bladder cancer.

**Materials and Methods**

**Cell lines and cell culture.** Six human bladder cancer cell lines were used: DSH1 (16), 8F/7 (17), RT112 (18), UMCUC-3 (19), SNU-14 (20), and T24 (21). The cell lines were cultured in RPMI 1640 (Sigma Chemical Co.) supplemented with 10% FCS, 12.5 mmol/L HEPES, and penicillin-streptomycin at 37°C in 5% CO2. 

**Cell invasion assay.** BD BioCoat Matrigel Invasion Chambers (Becton Dickinson) were used. Cells were suspended to a concentration of 1 × 10^7/mL in serum-free RPMI 1640. The cell suspension (500 μL) was added to the insert of the Matrigel-coated invasion chamber (24 well and 8-μm pore size) and incubated with RPMI 1640 with 10% FCS in the bottom of the chamber at 37°C in 5% CO2. Incubation time was modified according to the invasive ability of the cell lines. Noninvasive cells were removed by wiping with cotton swabs and the cells that attached to the lower surface of the membrane were fixed with 70% ethanol. Cells were then stained with hematoxylin and counted using a microscope (Eclipse E1000M, Nikon). Invasive cells were quantified by counting the number of cells in the five densest spots identified on the lowest face of the filter within a single ×200 field. Each experiment was done in triplicate.

**Processing of cell culture supernatants.** RT112 and T24 were grown to 70% to 80% confluence in RPMI 1640 with 10% FCS in 100-mm dishes. The cells were washed with PBS three and cultured in 10 mL serum-free RPMI 1640. In pilot experiments, we found that these cell lines proliferate for at least 2 d when grown in serum-free RPMI 1640 (data not shown). Twenty-four hours later, cell culture supernatants were collected from four dishes (40 mL) and centrifuged at 150,000 × g and 4°C for 30 min in a Beckman Optima XL-100 ultracentrifuge (Beckman Coulter) to remove cells and cell debris completely. The supernatants were then concentrated to ~1 mL (0.6 mg/mL) by using an Amicon Ultra centrifugal filter device (15 mL, 5K NMWL, Millipore). Protein in RPMI 1640 with 10% FCS was collected in the same way as the conditioned medium and used as a control. Total protein (120 μg) was fractionized with the ProteomeLab PF 2D Protein Fractionation System (Beckman Coulter). The separation was done using a PF2D HPRP column (Beckman Coulter) at 50°C with a flow rate of 0.75 mL/min. The column was equilibrated with solvent A [0.1% (v/v) trifluoroacetic acid in water] and eluted with solvent B [0.08% (v/v) trifluoroacetic acid in 100% (v/v) acetonitrile]. The gradient was 0% to 100% solvent B for 30 min. Eluent from column was automatically collected every 30 s into the 96-well plate with a fraction collector. Comparing the chromatogram profiles, fractions with peaks originating from FCS were excluded from further analysis. Fractions with specific peaks for cell culture supernatants were pooled into seven fractions, lyophilized, and resuspended in 100 μL of 20 mmol/L ammonium bicarbonate buffer (pH 8.0).

**Two-dimensional liquid chromatography-MS/MS and data analysis.** After trypsination of the fractions, two-dimensional liquid chromatography-MS/MS shotgun analysis was done by using an online nanoliquid chromatography system (Dina system, KYA Technologies) and a quadrupole time-of-flight mass spectrometer (Q-Tof Premier, Waters Micromass) as previously described (22). The identified proteins were obtained from MS/MS experiments by Mascot software (Matrix Science) using the Swiss-Prot human protein database containing 13,799 entries of human proteins (April 2006). The proteins were considered identified if they had resulting Mascot scores of >35 and at least two peptides with score >20. To determine the cellular localization of identified proteins, we used the information available from Swiss-Prot database.

**cDNA microarray.** Bladder cancer tissues for cDNA microarray analysis were obtained with informed consent from 55 patients who underwent surgery at our hospital. Tumor staging was assessed according to tumor-node-metastasis classifications (26 pTa, 11 pT1, 3 pT2, 13 pT3, and 2 pT4). Each bladder cancer sample was obtained by cold-cup biopsy at the time of transurethral surgery. As for the substitution of normal urothelium in the bladder, ureteral urothelium was obtained from 16 patients who had undergone nephrectomy for renal cell carcinoma. Ureteral urothelium was removed from the surgical specimen and the adjacent portion of the specimen was examined pathologically to confirm that neither urothelial dysplasia nor malignancy existed. Microarray experiments were done using a cDNA array containing ~30,000 50-mer oligonucleotides (MWG Biotech AG) fabricated by Pacific Edge Biotechnology Ltd. in New Zealand as described (23). In each hybridization, fluorescent cDNA targets were prepared from a tissue RNA sample and a reference RNA sample was derived from a pool of cell lines of different cancers. The
differences of gene expression profiles between T24 cells treated with anti-CXCL1 neutralizing antibodies or control IgG were examined by Human Genome U133 Plus 2.0 Array (Affymetrix, Inc.). cRNA preparation and hybridization to oligonucleotide arrays were according to Affymetrix protocol.

Reverse transcription-PCR. The primer sequences used for reverse transcription-PCR (RT-PCR) were as follows: CXCL1, 5′-CAAGGCCCGCTGTG-3′ (forward) and 5′-CCTTCTTGCCTGTCGACGTG-3′ (reverse); CXCR2, 5′-ATTICTGGGCATCTCCACAG-3′ (forward) and 5′-TGCACTTAGGCAACAGGAGTCT-3′ (reverse); matrix metalloproteinase-13 (MMP-13), 5′-TCGTAACGGCTTCTC-CCCTAAA-3′ (forward) and 5′-GCGATCTATATATACACATC-3′ (reverse); glyceraldehyde-3-phosphate dehydrogenase, 5′-GAAGGTGATGGGATTTC-3′ (forward) and 5′-CTTATGGGCTGTTCTGGTCAGTT-3′ (reverse); human CXCL1 cDNA was generated by RT-PCR using PuI polymerase (Stratagene) from total RNA isolated from T24. The primers used for amplification were the following: 5′-CAAGGCCCGCTGTG-3′ (forward) and 5′-CCTTCTTGCCTGTCGACGTG-3′ (reverse). The PCR product was ligated into XhoI/BamHI sites of pcDNA3.1(-) vector (Invitrogen). RT112 cells were transfected with CXCL1 cDNA using Lipofectamine 2000 (Life Technologies, Inc.). Single clones of the stable transfectants were selected with 900 μg/mL G418. Each clone was checked for expression of CXCL1 by RT-PCR and ELISA.

Establishment of stable transformants of CXCL1. The full-length human CXCL1 cDNA was generated by RT-PCR using PuI polymerase (Stratagene) from total RNA isolated from T24. The primers used for amplification were the following: 5′-CAAGGCCCGCTGTG-3′ (forward) and 5′-CCTTCTTGCCTGTCGACGTG-3′ (reverse). The PCR product was ligated into XhoI/BamHI sites of pcDNA3.1(-) vector (Invitrogen). RT112 cells were transfected with CXCL1 cDNA using Lipofectamine 2000 (Life Technologies, Inc.). Single clones of the stable transfectants were selected with 900 μg/mL G418. Each clone was checked for expression of CXCL1 by RT-PCR and ELISA.

Growth inhibition assay. RT112 and T24 cells (1×10⁴ and 4×10⁴, respectively) were seeded into 96-well plates in quintuplicate in RPMI 1640 with 10% FCS and allowed to adhere overnight. The cultures were then washed and refed with medium. Cells were incubated for 24 to 48 h. For treatment with neutralizing antibodies, monoclonal anti-CXCL1 or control antibody (mouse IgG; R&D Systems) was added to the medium. Proliferative activity was determined by the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay using a microtiter plate reader at 540 nm.

Measurement of urinary CXCL1 levels. Clean-catch urine specimens at clinic visits were collected throughout a period of several months from patients seen in our hospital. The control group consisted of outpatients attending with diseases of the urological tracts (n = 31, ages 48-84 y; benign prostatic hyperplasia in 14, prostate cancer in 9, lithiasis in 4, neurogenic bladder in 2, renal cancer in 1, and adrenal tumor in 1). Bladder cancer group consists of 67 patients (ages 47-90 y) before undergoing transurethral resection of bladder tumor for later histologically confirmed urothelial cancer (32 pTa, 23 pT1, and 12 pT2-4). There were no age differences between control group and bladder cancer group. These groups do not include the urine samples with >30 leukocytes/field. Collected samples were centrifuged for 10 min at 2,000 rpm at room temperature to remove debris, aliquoted, and stored -80°C. On the day of analysis, frozen urine samples were thawed quickly, and the urinary CXCL1 levels were measured using the Quantikine ELISA kit. Urine creatinine levels were measured spectrophotometrically using the alkaline picrate method.

Statistical analysis. The raw microarray data were analyzed using the nonparametric Mann-Whitney U test to compare the gene expression level between noninvasive pTa tumors and invasive pT1-4 tumors. The results of urinary CXCL1 levels were analyzed using the Mann-Whitney U test for two-group comparisons. The optimal sensitivity and specificity of the urinary CXCL1 levels for diagnosis of bladder cancer and for staging were determined by receiver operating characteristic (ROC) curve analysis using R statistical package. Univariable and multivariable logistic regression models were used to calculate odds ratios and 95% confidence intervals. Statistical analysis of the data was done using the StataView-1.0 program (Abacus Concepts, Inc.). A value of P < 0.05 was considered statistically significant.

Results

Evaluation of in vitro invasiveness of bladder cancer cell lines and their proteome in cell culture supernatant. We choose six different bladder cancer cell lines. Of them, three cell lines, DSH1, KU-7, and RT112 cells, are commonly used as models of superficial bladder cancer and UMUC-3, S637, and T24 cells are generally used as models of invasive bladder cancer. Then, we examined their invasion ability in vitro by using a modified Boyden chamber assay. As shown in Fig. 1A, the number of cells infiltrated was higher in the latter group of cells; this result

2 http://www.takara-bio.co.jp/
indicated that UMUC-3, 5637, and T24 are highly invasive, whereas DSH1, KU-7, and RT112 are poorly invasive tumor cells. DSH1 and RT112 showed almost no invasion ability and T24 cells exhibited the strongest invasive ability.

Because secreted proteins are thought to be suitable targets for the development of diagnostic markers and/or for identification of new drug targets (8, 9), we attempted to identify secreted proteins that were potentially involved or associated with the invasion of bladder cancer through the proteomic comparison of culture supernatants from highly invasive T24 and less invasive RT112 cells. As protein-rich FCS could easily mask proteins of interest in this analysis, we used serum-free medium as described previously (11, 12) and excluded contamination as far as possible by removing chromatogram fractions with peaks originating from FCS. As shown in Fig. 1B, fractions eluted from 11 to 23 min, which seemed to be produced from cultured cells, were pooled and analyzed by shotgun proteomics. A total of 477 proteins were identified in the culture supernatants of RT112 and T24 cells (218 and 295 proteins, respectively). Swiss-Prot database annotated the cellular localization of 330 proteins and 43 of them were predicted to be secreted proteins (Fig. 1C). Of these secreted proteins, 22 of them were detected only in highly invasive T24 cells (Supplementary Table S1).

**Up-regulation of CXCL1 in invasive bladder tumors in vitro and in vivo.** To further investigate the significance of these proteins in the invasion of bladder cancer, we compared the proteome data with mRNA expression profiles that consisted of 26 noninvasive pTa tumors and 29 invasive pT1-4 tumors in vivo (23). Among those 22 proteins specific for highly invasive T24 cells, there were corresponding probe sets for 14 of them on the custom-made chips. Of them, the expression of CXCL1 was significantly higher in invasive tumors than in noninvasive tumors in vivo (Fig. 2A). Additionally, the expression of this gene was more abundant in tumor tissues compared with that in normal uroepithelial cells. To confirm these results, the expression of this gene was examined in bladder cancer cell lines by using RT-PCR. It was revealed that all of the three highly invasive cell lines (UMUC-3, 5637, and T24) expressed CXCL1, whereas neither of them was detected in the less invasive DSH1, KU-7, and RT112 cell lines (Fig. 2B).

Because CXC chemokines are known to contribute to several tumor-related processes, such as tumor growth, angiogenesis/angiostasis, local invasion, and metastasis (25), we next examined the significance of CXCL1 in invasion of bladder cancer cell lines in vitro. First, we confirmed that all of the invasive cell lines secreted CXCL1 into the supernatants, whereas none of the less invasive cell lines secreted the detectable levels of this chemokine (Fig. 2C). These results indicate that higher secretion of CXCL1 is associated with the higher invasion ability of bladder cancer cells in vitro.

It was also confirmed that CXCR2, the gene for the receptor of CXCL1, was expressed in all of bladder tumor cell lines irrespective of their invasion ability (Fig. 2B). Because CXCL1 was highly expressed at the mRNA level in invasive bladder tumors in vivo, we next examined if this chemokine is also up-regulated at the protein level. Representative results of immunohistochemical staining are shown in Fig. 2D. It was revealed that positive staining was observed in none of the normal uroepithelium samples and in 40% of pTa tumors. On the other hand, as many as 75% of pT1-4 tumors stained positive. These observations led us to hypothesize that the expression of CXCL1 was related to tumor invasion of bladder cancer in vivo.

**Fig. 1.** A, invasion of bladder cancer cell lines in a modified Boyden chamber. Cells (5 \times 10^4) were seeded into the upper compartment of the chamber in serum-free RPMI 1640 and incubated for 24 h at 37°C. RPMI 1640 with 10% FCS filled the lower compartment. Columns, mean of the number of cells in the five densest spots identified on the lower surface of the filter within a single \( \times 200 \) field in each of three experiments; bars, SD. B, reverse-phase chromatograms of cell culture supernatants. Eluted proteins were detected by UV absorbance at 214 nm. In T24 and RT112 supernatant, peaks originating from FCS are indicated by arrows. The fractions from 11 to 23 min were collected for two-dimensional liquid chromatography-MS/MS analysis. C, schematic flow diagram depicting the steps for identifying proteins associated with invasion by human bladder cancer.
Secreted CXCL1 is sufficient for increased invasion by bladder cancer cells. Next, we clarified whether CXCL1 regulated the invasive ability of bladder cancer cells in vitro. We first examined the effect of knockdown of CXCL1 in highly invasive T24 cells by using RNAi vectors. We have established two different polyclones in which the expression of CXCL1 is reduced by 30% compared with mock-transfected control cells (Fig. 3A). It was revealed that this inhibition of CXCL1 expression decreased the number of infiltrated cells in the invasion assay to 30% (Fig. 3B). Because the treatment of T24 cells with anti-CXCL1 neutralizing antibodies resulted in comparable reduction in the number of infiltrating cells, these results suggest that secreted CXCL1 regulates the invasive ability of bladder cancer cells via an autocrine loop (Fig. 3C). As CXCL1 has previously been shown to stimulate cell proliferation as an autocrine growth factor in several cancer cell lines (26, 27), we examined the effect of this chemokine on cell proliferation. As shown in Fig. 3D, the knockdown of CXCL1 or its neutralization with specific antibodies showed no direct effect on proliferation of T24 cancer cell lines. These results indicated that secreted CXCL1 in the supernatant promotes the invasion of T24 cells but has little effect on the proliferation of these cells.

To examine if the expression of CXCL1 is sufficient to promote the enhanced invasion of bladder cancer cell lines, less invasive RT112 cells were stably transplanted with a plasmid that encoded CXCL1 and two independent monoclonal cultures were established. Both monoclonal successfully secreted large amounts of CXCL1 and showed higher invasive ability compared with mock-transfected control cells (Fig. 3E). These results indicated that the expression of CXCL1 is sufficient to promote the enhanced invasion of bladder cancer cells in vitro. As observed for T24 cells, this effect was suppressed by treatment with anti-CXCL1 neutralizing antibodies and the forced expression of this chemokine had no effect on the proliferation of RT112 cells (data not shown).

CXCL1 regulated the expression of MMP-13 in bladder cancer cells in vitro and the expression of CXCL1 was associated with that of MMP-13 in vivo. To clarify the mechanisms by which CXCL1 regulates the invasive ability of bladder cancer, we compared the mRNA expression profiles of highly invasive T24 cells treated with or without an anti-CXCL1 neutralizing antibody using the Affymetrix U133 Plus 2.0 Array. Among the 38,500 transcripts analyzed, we identified 62 RNAs whose abundance was decreased (<0.6-fold) by treatment with an anti-CXCL1 neutralizing antibody (Supplementary Table S2). It was revealed that a member of the MMP family, MMP-13, was down-regulated by the inhibition of CXCL1. To confirm this result, we examined the expression of MMP-13 in T24 and RT112 cells in which CXCL1 was engineered to be repressed or overexpressed, respectively. It was revealed that the expression of MMP-13 was regulated by the expression of CXCL1 in both cell lines (Fig. 4A). Because it has been reported that the expression of MMP-13 is associated with the invasion of bladder cancer in vivo (24), we next examined if the up-regulation of CXCL1 is associated with the higher expression of MMP-13 in bladder cancer cells in vivo. Positive staining of MMP-13 was observed in none of 11 tumors (6 with pTa, 4 with pT1, and 1 with pT3) with negative staining of CXCL1, whereas it was observed in 9 (1 with pT1, 7 with pT3, and 1 with pT4) of 19 (4 with pTa, 5 with pT1, 1 with pT2, 7 with pT3, and 2 with pT4) tumors with positive staining of that chemokine (Fig. 4B). It was revealed that statically significant correlation was observed between the expressions of both proteins ($P = 0.0064$, $\chi^2$ test). These results suggest the possibility that MMP-13 is also regulated by CXCL1 in vivo.

Fig. 2. A, cDNA microarray data for CXCL1. The normalized expression units of this gene were transformed into the log2 values. Bars of the box extend from the 25th to 75th percentile of the data and line in the middle represents the median. The upper and lower bars represent the distance from the 10th to 90th percentile from the median. The Mann-Whitney U test was used to compare expression levels between the two groups. B, expression of CXCL1 and CXCR2 mRNA by bladder cancer cell lines with different levels of invasiveness. The PCR conditions are as follows: denaturation (95°C, 5 min), 32 cycles of denaturation (94°C, 1 min), annealing (58°C, 1 min), and extension (72°C, 1.5 min). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Values were corrected for the total protein in the cell lysate. C, expression of CXCL1 protein by bladder cancer cell lines. Protein levels in cell culture supernatants were analyzed by ELISA. Each cell line was plated in triplicate; experiments were repeated at least twice. Bars, SD. D, immunohistochemical staining for CXCL1 in surgical tissue specimens. A brown precipitate in the cytoplasm denotes positive signal. Representative samples of negative (a, normal uroepithelium; b, pTaG1) and positive (c, pT2G3; d, pT3G3) staining are shown. Bars, 100 μm.
The level of CXCL1 in the urine reflects the existence of bladder cancer. Because immunohistochemical analysis showed that bladder tumors express CXCL1 in vivo, we determined if an increase of CXCL1 in the urine could indicate the existence of bladder cancer, especially that with invasive phenotype. The amount of CXCL1 in the urine was examined in 67 patients with histopathologically proven bladder cancer and 31 controls. Because it was shown that several chemokines are elevated in patients with febrile urinary tract infections (28), we eliminated urine samples contaminated with >30 leukocytes per microscopic field. The concentration of CXCL1 in the urine was examined by ELISA and corrected by the urinary creatinine level (Fig. 5A). The mean corrected CXCL1 level in the control group and in patients with noninvasive and invasive tumors was 7.8 ± 2.5, 17 ± 3.7, and 112 ± 25 pg/mg creatinine (mean ± SE), respectively. It was revealed that the corrected CXCL1 level from both noninvasive pTa tumors and invasive pT1-4 tumors was significantly higher than those in the controls (P = 0.0076 and P < 0.0001, respectively). More importantly, a significant difference was observed between noninvasive and invasive tumors (P = 0.0028). These results suggested that the corrected CXCL1 level in the urine would predict the existence of both noninvasive and invasive bladder tumors. So, we have done the ROC curve analysis to estimate the optimal cutoff point. Using 9.5 pg/mg creatinine as the cutoff value to predict the existence of noninvasive or invasive bladder cancer, measurement of corrected CXCL1 had a sensitivity of 57.1% and a specificity of 90.6% (area under the curve, 0.713; Fig. 5C). Finally, we have done multivariate stepwise logistic regression analysis incorporating CXCL1 as a continuous variable, cytology, age, and sex to examine that the corrected CXCL1 level could be an independent factor for predicting the invasive bladder cancer. It was revealed that cytology (odds ratio, 21.82; 95% confidence interval, 3.981-113.780; P = 0.003) and CXCL1 (odds ratio, 1.023; 95% confidence interval, 1.001-1.044; P = 0.038) were independent factors for predicting the bladder cancer with invasive phenotype (Table 1).

Discussion

Tumor cells overexpress many secreted proteins; some of these can be detected in serum samples from patients with cancer and have been considered as potential new tumor markers (29). Well-known secreted proteins that are used as biomarkers include a-fetoprotein for liver cancer or nonseminomatous germ cell tumors, as well as prostate-specific antigen for prostate cancer (30). Our previous analysis of serum-free cell culture supernatants showed that proteins in the culture supernatant reflected those produced by tumor cells in vivo (11). This observation strongly suggests that systematic analysis of the proteins secreted by cultured cancer cells will contribute to the identification of potential diagnostic and prognostic tumor biomarkers.

We compared the protein profile between highly invasive (T24) and less invasive (RT112) bladder cancer cell lines.
Candidate proteins were further screened using the cellular localization information from the Swiss-Prot database. Consequently, CXCL1 was significantly higher in invasive tumors than in noninvasive tumors in vivo (Fig. 2A).

CXCL1 is a member of the CXC chemokine family (31). This family of molecules can be classified according to the presence or absence of three amino acid residues (Glu-Leu-Arg; ELR motif) that precede the first cysteine amino acid residue in the primary structure (32). The ELR+ CXC chemokines (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8) are chemoattractants for neutrophils and are also potent angiogenic factors (33–35). In contrast, ELR- CXC chemokines (CXCL4, CXCL9, and CXCL10) are chemoattractants for mononuclear cells and are potent inhibitors of angiogenesis (35, 36). In a variety of human cancers, the ELR+ CXC chemokines have been found to be associated with tumorigenesis, angiogenesis, and metastasis (25, 37). The biological functions of ELR+ CXC chemokines are primarily mediated via CXCR2, a seven-transmembrane G protein–coupled receptor. CXCL1 protein was originally purified from the culture supernatant of Hs29T melanoma cells and is also known as melanoma growth stimulatory activity (38, 39). Although the elevated expression of CXCL1 has been reported in a series of human tumors (40, 41), the role of this chemokine in bladder cancer is poorly understood. In this study, we showed that higher expression of CXCL1 was associated with the invasive phenotype of bladder cancer both in vitro and in vivo (Fig. 2). We also showed that the secreted CXCL1 was sufficient for the invasion of bladder tumors in vitro (Fig. 3). Because the CXCL1 receptor CXCR2 is expressed in all the bladder cancer cell lines (Fig. 2B) and in most of the tumor tissues examined irrespective of the invasion phenotype (microarray analysis; data not shown), it is probable that secreted CXCL1 is associated with the invasion of the bladder cancer via an autocrine loop involving its receptor.

CXCL1 did not induce bladder cancer cell proliferation, although it has been shown to promote cell proliferation in several types of tumors. Its interaction with CXCR2 is shown to induce extracellular signal-regulated kinase 1/2 phosphorylation, which then leads to induction of EGR1, and these are responsible for increased cell proliferation (37). Therefore, we examined the extracellular signal-regulated kinase 1/2 pathway in T24 and RT112 cells in which CXCL1 was engineered to be repressed or overexpressed, respectively. It was revealed that CXCL1 did not induce this pathway in bladder cancer cells (data not shown).

As for the mechanisms by which CXCL1 regulates the invasive ability of bladder cancer cells, we found for the first time that this chemokine induced MMP-13 in vitro (Fig. 4A). Although it is still uncertain if this is also true in vivo, a recent study showed that MMP-13 is highly expressed in invasive bladder tumor tissue (24), supporting our preliminary immunostaining results (Fig. 4B). As for other MMPs, Zhou et al. (42) reported that a glioma cell line overexpressing CXCL1 showed an increase in motility and invasiveness and that CXCL1-transfected cells showed increased expression of MMP-2. However, the expression of MMP-2 was not affected by the introduction of CXCL1 in RT112 cell lines (data not shown). CXCL1 may regulate the invasion of tumors through several types of MMPs. It has also been reported that this chemokine regulates the expression of several proteins, such as β1 integrin, SPARC (42), vascular endothelial growth factor, and angiopoietin-2 (43). So, several factors including MMP-13 are likely to be involved in CXCL1-mediated regulation of bladder cancer invasion.
Many studies have focused on the detection of specific bladder cancer–associated proteins in the urine of patients. Thus far, BTA, BTA stat, NMP22, and fibrinogen degradation products have been used as commercial diagnostic markers for bladder cancer in the urine (44). In addition, a lot of soluble urine marker is reported (45, 46). However, none of these markers is sensitive enough for routine clinical use (44). Recently, several investigators have proposed that high-throughput technologies, such as gene expression microarrays or proteomics, may be a new way to identify biomarkers for urothelial cancer in the urine (7, 47), and it is expected that this technology will be more widely used to identify novel cancer biomarkers in the future. In the present study, we showed for the first time that the level of CXCL1 was elevated in the urine from patients with bladder cancer (Fig. 5). These results indicate that an elevated CXCL1 in the urine could predict the existence of bladder cancer, especially in patients with invasive disease. Our results suggest that measurement of the level of CXCL1 in urine may be a useful biomarker for the early detection of invasive bladder cancer, and the inhibition of signals through CXCL1 might be a potent therapeutic target for preventing the progression of the bladder cancer. Additionally, CXCL8, one of ELR CXC chemokines that share 44% amino acid sequence identity with CXCL1 and are reported to be elevated in the urine of patients with transitional cell carcinoma (48). Further prospective and comparative studies in a different population are required for the precise evaluation of diagnostic values of CXC chemokines in bladder cancer.

Table 1. Multivariable stepwise logistic regression analyses of CXCL1, cytology, age, and sex for prediction of invasive stage (≥T1)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds ratio (95% confidence interval)</th>
<th>P</th>
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<tr>
<td>CXCL1*</td>
<td>1.023 (1.001-1.044)</td>
<td>0.038</td>
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<tr>
<td>Cytology (positive vs negative)</td>
<td>21.82 (3.981-113.7)</td>
<td>0.003</td>
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*Urinary CXCL1 levels were analyzed as continuous variables.

Fig. 5. A, CXCL1 levels in the urine of controls and patients with bladder cancer. Values were corrected by the urine creatinine concentration. Points, mean; bars, SE. B, ROC curve analysis of the urinary CXCL1 levels. The optimal cutoff of the urinary CXCL1 levels for distinguishing bladder tumor from control was determined as 9.5 using the ROC curve. C, ROC curve analysis of the urinary CXCL1 levels for distinguishing invasive from noninvasive bladder tumor. For all patients with bladder tumor, the optimal cutoff value of the urinary CXCL1 levels for distinguishing invasive from noninvasive bladder tumor was determined as 37.5 using ROC curve analysis.
The role of chronic inflammation in cancer progression continues to gain importance in the biological events for several types of cancers. Recurrent or persistent inflammation may induce, promote, or influence susceptibility to carcinogenesis by causing DNA damage, initiating tissue reparative proliferation, and/or creating a stromal “soil” that is enriched with cytokines and growth factors (49). The evidence for an association between chronic inflammation and squamous cell carcinoma of the bladder is strong and widely accepted. In transitional cell carcinoma of the bladder, several animal and human studies strongly support the hypothesis that chronic inflammation induced by persistent urinary tract infections plays a role in the bladder carcinogenesis (50). As CXC chemokines, including CXCL1, play major roles in inflammation and wound healing (25). So, further understanding of roles of these chemokines on the tumorigenesis and disease progression of bladder cancer will be required to provide a new rationale for targeted therapy for this tumor.

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References

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