IL-5-induced migration via ERK1/2-mediated MMP-9 expression by inducing activation of NF-κB in HT1376 cells

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Abstract. Interleukin-5 (IL-5) plays an important role in the growth and differentiation of human B cells and eosinophils. However, little is known about the effect of IL-5 on cancer cells. In this study, we investigated the molecular mechanisms involved in the IL-5-induced migration of HT1376 bladder cancer cells. Our results indicated that IL-5 significantly enhanced migration and MMP-9 expression in HT1376 cells. We also found that IL-5 induces transcriptional activation of the binding of NF-κB and AP-1, which are two important nuclear transcription factors that are linked to MMP-9 expression in HT1376 cells. In subsequent experiments, we found activation of ERK1/2 in IL-5-treated HT1376 cells. To examine the involvement of the ERK1/2 signaling pathway on IL-5-induced cell responses, we pretreated HT1376 cells with the ERK1/2 inhibitor U0126 followed by IL-5 treatment. The results showed that U0126 treatment inhibited migration of IL-5-treated HT1376 cells. Moreover, IL-5-stimulated MMP-9 expression was suppressed by the addition of U0126. Inhibition of ERK1/2 function consistently rescued transcriptional activity of NF-κB, without altering AP-1 activation, in IL-5-treated cells. Finally, inhibition of the IL-5-specific receptor IL-5Rα by small interfering RNA (siRNA) suppressed migration, ERK1/2 activation, MMP-9 expression and binding activation of NF-κB in IL-5-treated HT1376 cells. The results of the present study indicate that the IL-28A/IL-28AR1 dyad induces cell migration through ERK1/2-mediated expression of MMP-9 by binding activation of NF-κB in bladder cancer cells. In conclusion, these novel findings indicate that binding of IL-5 to IL-5Rα plays a critical role in MMP-9 expression, which may be involved in the migration of bladder cancer.

Introduction

Bladder cancer is the most common form of cancer in developed countries. The vast majority of malignant tumors found in the urinary bladder are transitional cell carcinoma (TCC) (1), which is a type that is intricately associated with metastasis (2). The knowledge of the cellular and molecular mechanisms of metastasis in TCC of the bladder is essential for the potential application of effective treatment.

The matrix metalloproteinase (MMP) family of extracellular proteinases plays a central role in the migration and invasion of tumor cells (3,4). Tumor cell migration requires a degradation of the extracellular matrix (ECM) and basement membrane by proteases such as MMP-2 (72 kDa) and MMP-9 (92 kDa) (3,4). Previous results demonstrated that MMP-9 expression is associated directly with tumor grade, invasion, migration, and metastasis in the progression of bladder cancer (5-10). MMP-9 is induced by several growth factors and cytokines in different cell types (11-13). Accumulative studies showed that the identification of the transcription factors, including NF-κB, Sp-1 and AP-1, was essential for the induction of MMP-9 in cancer cells (11-13).

IL-5 is a T-cell replacing factor (TRF) that stimulates the differentiation of B cells (14). Previous studies have demonstrated the regulatory roles involved in the activation, proliferation and survival of eosinophils (15). IL-5 binds at the cell surface of a receptor made up of heterodimer complexes composed of 2 chains: ligand-specific receptor IL-5Rα and the accessory receptor β-subunit (βc) (14,15). The binding of IL-5 to IL-5Rα resulted in the activation of Jak/Stat, MAPK and PI3K in B cells and eosinophils (14,15). Although many studies have demonstrated the biological role of IL-5, its exact regulatory mechanism in the process of tumor cell migration remains unknown.

Here, we show the molecular and cellular mechanism involved in cytokine IL-5-induced cell migration. In the present study, the expression of both IL-5 and its receptor IL-5Rα was observed in bladder cancer HT-1376 cells. This study is the first to show the potent induction of cell migration by IL-5 in bladder cancer cells. In addition, our results demonstrated the essential role of ERK1/2-mediated MMP-9 expression in IL-5-induced migration of bladder cancer cells.

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Materials and methods

Materials. Polyclonal antibodies to ERK, phospho-ERK, p38MAPK, phospho-p38MAPK, JNK and phospho-JNK were obtained from Cell Signaling (Danvers, MA). U0126 was obtained from Calbiochem (San Diego, CA). The polyclonal MMP-9 antibody was obtained from Chemicon. Small interfering RNA (siRNA) oligonucleotides targeting IL-5Rα (5'-GCCAGAACGACCACTACTA-3') and scramble (5'-CUGUCAGUCGUAGUAAU-3') were designed and synthesized by Genolution (Seoul, Korea).

Cell cultures. A human bladder carcinoma cell line (HT1376) was obtained from the American Type Culture Collection. The cells were maintained in DMEM (4.5 g glucose/liter) supplemented with 10% fetal calf serum, L-glutamine and antibiotics (Biological Industries, Beit Haemek, Israel) at 37°C in a 5% CO₂ humidified incubator.

RNA extraction. Total RNA was isolated from tissue using TRIzol reagent (Life Technologies, Grand Island, NY), according to the manufacturer's protocol. The quality and integrity of the RNA was confirmed by agarose gel electrophoresis and ethidium bromide staining, followed by visual examination under ultraviolet light.

Real-time PCR. Real-time PCR assays using a Rotor-Gene 3000 PCR system (Corbett Research, Mortlake, Australia) were performed in the original and independent cohorts. GAPDH was analyzed in parallel as an endogenous RNA reference gene, and data were normalized to the expression of GAPDH.

Immunoblot. Growth-arrested cells were treated with IL-5 in the absence of 10% FBS for various durations at 37°C. The cells were then washed twice with cold PBS and freeze-thawed in 250 µl lysis buffer (containing, in mmol/l, HEPES (pH 7.5) 50, NaCl 150, EDTA 1, EGTA 0.5, DTT 1, β-glycerophosphate 10, NaF 1, NaVO₃ 0.1, and phenylmethylsulfonyl fluoride 0.1 and 10% glycerol, 0.1% Tween-20, 10 µg/ml of leupeptin and 2 µg/ml of aprotinin), and then scraped into 1.5-ml tubes. The lysates were placed on ice for 15 min and then centrifuged at 12,000 rpm for 20 min at 4°C. The protein concentration of the supernatant was determined using a Bradford reagent at 12,000 rpm for 20 min at 4°C. The protein concentration was rinsed with water, dried and exposed to X-ray film over night.

Wound healing migration assay. Cells were plated on 6-well dishes and grown to 90% confluency in 2 ml of growth medium. The cells were damaged using a 2-mm-wide tip and were then treated with IL-5. They were allowed to migrate, and photographs were taken through an inverted microscope (original magnification, x40).

Statistical analysis. Where appropriate, data were expressed as the mean ± SE. Data were analyzed by factorial ANOVA and Fisher's least significant difference test where appropriate. Statistical significance was set at P<0.05.
Results

Expression of IL-5 and its receptor IL-5Rα in bladder cancer HT1376 cells. To investigate whether the IL-5 and its receptor IL-5Rα is expressed in bladder cancer HT1376 cells, we analyzed IL-5 and IL-5Rα mRNA expression. Real-time PCR analysis showed detection of mRNA expression of IL-5 in HT1376 cells (Fig. 1A). Expression of IL-5Rα mRNA was also observed in HT1376 cells (Fig. 1A). Moreover, both IL-5 and IL-5Rα mRNA expressions were strongly enhanced by the addition of 10% FBS (Fig. 1A). These results indicate that expression of IL-5 and its receptor IL-5Rα can be found in bladder cancer HT1376 cells.

IL-5 induces wound healing migration and MMP-9 expression in HT1376 cells. Previous studies have demonstrated the involvement of cell migration in the development of bladder cancer (1,2). We next examined the effect of IL-5 on bladder cancer cell migration using a wound healing migration assay. Treatment of HT1376 cells with IL-5 for 24 h induced the capacity of migration, as compared with the control (Fig. 1B). To explore the relationship between cell migration and MMP expression, we performed gelatin zymographic assay in IL-5-treated HT1376 cells. IL-5 was added to HT1376 cells at various concentrations, in order to determine the optimal dose. IL-5 significantly induced both MMP-2 and MMP-9 expression at 100 ng/ml, as compared with the control in HT1376 cells (Fig. 1C). In addition, both MMP-2 and MMP-9 levels were increased in IL-5-treated HT1376 cells in a time-dependent manner (Fig. 1C). The effect of IL-5 was confirmed by immunoblot. Increase in expression levels of both MMP-2 and MMP-9 were observed in HT1376 cells induced by IL-5 (Fig. 1C).

IL-5-induced MMP-9 expression is involved in binding activities of NF-κB and AP-1 in HT1376 cells. Since the expression of MMP-9 is deeply associated with the development of bladder cancer (5-10), we focused our investigation on IL-5-induced MMP-9 expression. In order to define transcription factors involved in the IL-5 induction of MMP-9 in HT1376 cells, we performed a gel shift assay (EMSA) using nuclear extract obtained from cells cultured for 24 h in the presence or absence of IL-5 (100 ng/ml). IL-5 significantly increased nuclear binding to NF-κB and AP-1 binding sites.
However, no significant Sp-1 levels were observed in IL-5-treated HT1376 cells (Fig. 2). These results suggest that IL-5-induced MMP-9 expression might be mediated through the binding activation of NF-$\kappa$B and AP-1.

ERK1/2 inhibitor, U0126, decreases the IL-5-induced migration in HT1376 cells. To examine the MAPK signaling pathway in IL-5-treated HT1376 cells, we carried out immunoblot experiments. Treatment with IL-5 resulted in a significant induction of ERK1/2 activation in HT1376 cells (Fig. 3A). IL-5-induced activation of ERK1/2 was suppressed by U0126, ERK1/2-specific inhibitor (Fig. 3B). In contrast, treatment of HT1376 cells with IL-5 did not lead to activation of JNK and p38MAPK (Fig. 3A). To further investigate the role of ERK1/2 signaling in IL-5-treated HT1376 cells, cells were pretreated with U0126. Blockage of ERK1/2 signaling significantly reduced the migration of HT1376 cells induced by IL-5 (Fig. 4A). These results indicate that ERK1/2 signaling plays an important role in the IL-5-induced migration of bladder cancer cells.

Inhibition of ERK1/2 signaling abolishes IL-5-mediated MMP-9 expression and NF-$\kappa$B activation in HT1376 cells. The results of the present study showed that IL-5 regulates cell migration and MMP-9 expression (Fig. 1B and C). In addition, IL-5-mediated migration of bladder cancer cells was suppressed by an ERK1/2 specific inhibitor U0126 (Fig. 4A). Thus, to define the role of ERK1/2 signaling in IL-5-induced MMP-9 expression, HT1376 cells were pretreated with U0126 (10 µM) for 40 min followed by stimulation with IL-5 (100 ng/ml) for 20 min, and the immunoblot was measured for the activation levels of ERK1/2.

Knockdown of IL-5Rα, ligand-specific IL-5 receptor, reduces IL-5-induced migration of HT1376 cells. To determine the regulatory mechanism of IL-5, we used the siRNA-mediated knockdown of IL-5Rα in IL-5-treated HT1376 cells. The cells were transfected with si-IL-5Rα and scramble siRNA, respectively, followed by treatment with IL-5. To evaluate the transfection efficiency of siRNA in HT1376 cells, expression of IL-5Rα was examined by immunoblotting. IL-5Rα was inhibited by transfection of HT1376 cells with a specific siRNA against IL-5Rα (si-IL-5Rα) (Fig. 5E). In addition, transfection of scramble siRNA into cells remained unchanged for protein...
Figure 4. Effects of U0126 on wound-healing migration, MMP-9 expression, and binding activity of NF-κB in IL-5-treated HT1376 cells. (A) After serum starvation for 24 h, cells were pre-incubated with U0126 (10 µM) for 40 min, and stimulated by the addition of IL-5 (100 ng/ml) for 24 h, and wound healing migration assay was performed, as described in Materials and methods. (B) The cells were treated with IL-5 (100 ng/ml) in the presence or absence of the U0126 (10 µM). The cultured medium was analyzed for the expression of MMP-9 using zymographic assay. Immunoblot assay was used to determine the cell lysates by measuring the protein levels of MMP-9. (C) EMSA analyses were performed from nuclear extracts for the activated NF-κB and AP-1 using radiolabeled oligonucleotide probes.

Figure 5. Blockage of IL-5Rα reversed increased wound healing migration, ERK1/2 activation, MMP-9 expression, and NF-κB binding activity in the IL-5-mediated stimulation of HT1376 cells. (A) Cells were transfected with either si-IL-5Rα or scramble siRNA for 24 h, followed by stimulation with IL-5 (100 ng/ml), and wound healing migration was measured after 24 h, as described in Materials and methods. (B) Cells were transfected with either si-IL-5Rα or scramble siRNA for 24 h, further stimulated with IL-5 (30 ng/ml) for 20 min, and the activation levels of ERK1/2 were determined by immunoblotting. (C) Cells were transfected with either si-IL-5Rα or scramble siRNA for 24 h, followed by stimulation of IL-5. After 24 h, zymographic and immunoblot analyses for MMP-9 were determined in the cultured medium and cell lysates. (D) Nuclear extracts from the cells were analyzed by EMSA for the binding activity of NF-κB using radiolabeled oligonucleotide probes. (E) Effect of IL-5Rα silencing genes in HT1376 cells. Cells were transfected with either si-IL-5Rα or scramble siRNA. After 24 h, protein level of IL-5Rα was observed by immublot analysis.
levels of IL-5Rα (Fig. 5E). The results suggested that the siRNA molecule was specific and effective. The effect of si-IL-5Rα was then determined on IL-5-induced migration. Our results showed that the inhibition of IL-5Rα significantly reduced the migration of HT1376 cells induced by IL-5, compared to siRNA control groups (Fig. 5A). These data suggest that IL-5 induced the migration of bladder cancer cells through its specific IL-5 receptor, IL-5Rα.

Inhibition of IL-5Rα knockdown decreases ERK1/2 activation, MMP-9 expression, and binding activity of NF-κB in IL-5-treated HT1376 cells. We sequentially further investigated the effects of si-IL-5Rα on the induction of ERK1/2 activation, MMP-9 expression, and binding activity of NF-κB in IL-5-treated HT1376 cells. As shown in Fig. 5B, transfection of si-IL-5Rα significantly suppressed the activation of ERK1/2 in response to IL-5. In addition, the blockade of IL-5Rα reversed MMP-9 expression and NF-κB binding activity to control levels in IL-5-treated HT1376 cells (Fig. 5C and D). These results suggest that IL-5 enhanced ERK1/2 activation, MMP-9 expression, and binding activity of NF-κB via IL-5Rα receptor in HT1376 cells.

Discussion

Although the role of cytokine IL-5 in the biological responses of immune cells is well established, the role and mechanism involved in IL-5-induced migration of tumor cells remains to be investigated. Previous studies proposed that IL-5 is a regulatory cytokine supporting the growth and differentiation of activated B cells (14). Subsequent studies have shown the essential roles of IL-5 in the growth, activation and survival of eosinophils (15). Some studies have indicated that IL-5 has an antitumor effect in mouse B cell lymphoma and colon tumor cells (17,18). In contrast, the results of the present study from our results showed that IL-5 plays a pivotal role in the migration of bladder cancer cells.

The inflammatory process may be responsible for the development and progression of cancer (19). Inflammation in the bladder is the result of several pathological processes, which involve the accumulation of immune cells and the release of cytokines (20,21). We hypothesize that increased production of inflammatory cytokines may contribute to an altered microenvironment in the bladder, which leads to the progression of bladder cancer. In the first stage, real-time PCR analysis revealed that the mRNA expression of IL-5 and its specific subunit receptor IL-5Rα were detected in bladder cancer HT1376 cells. These results suggest that IL-5 is constitutively expressed in bladder cancer cells and might be an important regulatory cytokine associated with the progression of bladder cancer.

We next investigated the molecular regulation involved in the development and progression of bladder cancer. In the present study, we demonstrated that IL-5 enhanced wound healing migration of bladder cancer cells. In addition, both MMP-2 and MMP-9 expressions were induced by IL-5 treatment. MMPs have been implicated in cell migration through the degradation of extracellular matrix components (3,4). It is well accepted that MMP-2 and MM-9 are particularly important factors in cell migration (3,4). The importance and role of MMP-9 in bladder tumor has been demonstrated in in vivo orthotopic xenograft models (22,23), preclinical evidence (5-9), and in the study of polymorphisms (24). Therefore, we investigated the transcription factors binding to human MMP-9 promoter regions. Several proximal binding sites, including NF-κB, AP-1 and Sp-1, that regulate MMP-9 promoter have been identified in tumor cells (11-13). In 5637 bladder cancer cells stimulated with TNF-α, NF-κB was shown to be essential for MMP-9 expression, but AP-1 and Sp-1 was not affected (25). Our EMSA results indicated that IL-5 increased the binding activity of NF-κB and AP-1, known binding sites in the MMP-9 promoter, without detecting inducible levels of Sp-1. We concluded that NF-κB and AP-1 sites may be responsible for the IL-5-induced transcriptional activation of the MMP-9 in HT1376 bladder cancer cells.

Several signaling pathways have been identified in the MMP-9 regulation of various cells (16,26-29). Studies of MMP-9 in bladder cancer cells have focused mainly on investigating the signaling pathways in response to various factors. The ERK1/2 pathway mediates induction of MMP-9 via TNF-α in HT1376 cells (30). The involvement of p38MAPK has been associated with the regulation of MMP-9 expression in bladder cancer HTB9, HTB5, 5637 and HT1376 cells (10,25,30). In a recent study, Ras-induced MMP-9 expression was inhibited by RhoA inhibitor Y-27632 (31). However, the issue of the signaling pathways underlying the induction of MMP-9 in IL-5-treated cancer cells has not yet been clarified. In the present study, our result showed that IL-5 treatment induced activation of ERK1/2 in HT1376 cells. Our data also show that U0126 (ERK1/2 inhibitor) decreases IL-5-induced wound healing migration and MMP-9 expression via reductions in NF-κB binding without altering AP-1 activation in HT1376 cells. The results of the present study clearly show that ERK1/2 mediates MMP-9 expression via activation of NF-κB binding, which results in enhanced cell migration in bladder cancer HT1376 cells. It is possible that NF-κB-mediated expression of MMP-9, regulated by ERK1/2, may cooperate in the migration of bladder cancer cells.

IL-5 transduces signals through heterodimer receptor complexes, a unique IL-5Rα and a common β-subunit (βc), leading to the activation of different signaling pathways on target cells (14,15). Previous reports have suggested that the cytoplasmic region of the IL-5Rα subunit is critical for IL-5 signaling (14,15,32). We therefore investigated whether IL-5Rα contributes to the IL-5-mediated cell responses using IL-5Rα-specific siRNA (si-IL-5Rα) in bladder cancer cells. The increased migration, activation of ERK1/2, MMP-9 expression, and NF-κB binding activity in response to IL-5 was suppressed by abolition of IL-5Rα, suggesting that IL-5Rα is indispensable in the transmission of the migratory cell signal of IL-5.

Our results disagree with those of previous reports showing an antitumor effect of IL-5 on mouse B cell lymphoma and colon tumor cells. Considering these opposing results, this may explain the differences in cell responses by IL-5 within tumor cell type species. Although an emerging amount of attention is being paid to the opposing functions of inflammatory cytokines as extrinsic suppressors of tumors and as pro-tumor growth stimulators, the model remains to be controversial (19,33). In the present study, we showed that IL-6 released by
bladder cancer cells could play a crucial role in tumor growth and development. Further studies are required to investigate the role of IL-5 in tumor growth using animal models.

In summary, our results are consistent with 3 major results: i) both IL-5 and IL-5Rα are produced by bladder cancer HT1376 cells; ii) IL-5-induced migration regulates ERK1/2-mediated MMP-9 expression via the binding activity of NF-κB in HT1376 cells; and iii) IL-5Rα receptor is essential for the migration of bladder cancer HT1376 cells by IL-5, which may be mediated in part by regulating the ERK1/2-associated MMP-9 expression via activation of NF-κB binding. Collectively, these novel results point to the potential use of IL-5 in future potential molecular therapy for bladder cancer.

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