Interleukin-18 increases metastasis and immune escape of stomach cancer via the downregulation of CD70 and maintenance of CD44

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doi:10.1093/carcin/bgp158
Advance Access publication July 28, 2009

Introduction

Stomach cancer is one of the most malignant and commonly occurring cancers in the world, especially in East Asia (1). It has been suggested that several factors are involved in the development of stomach cancer. One of the well-known causative factors is Helicobacter pylori infection, which causes gastritis and increases the risk of developing stomach cancer (2,3). It is believed that more than half of mankind is infected with H.pylori, and >70% of Korean adults are infected (4). Stomach cancer is the second most common cause of cancer-related death and its clinical response is highly dependent on cancer stage (5,6). However, its cure rate is low because the majority of stomach cancers are malignant when diagnosed and the incidences of local invasion and metastasis are high.

CD70 is costimulatory molecule, which belongs to the tumor necrosis factor (TNF) family and its ligand is CD27, which is found on T or natural killer (NK) cells (7,8). It is generally known that CD70 is highly expressed on Epstein-Barr virus-transformed lymphoblastoid cells and human immunodeficiency virus-infected T cells and that this expression can be induced by some cytokines like TNF-α (9,10). CD70 has been reported to have diverse effects as a result of its interaction with its receptor. On B cells, CD70 can promote the differentiation of memory B cells into plasma cells and this process is synergistically augmented by the addition of interleukin (IL)-10 (11,12). Moreover, many studies have found that the CD27–CD70 interaction can serve as a second signal for T cell activation and TNF-α production (13,14). In addition, this interaction can induce the activation of NK cells and increase their killing activity (7,15). Based on the effect of the CD27–CD70 interaction on T and NK cells, an attempt was made recently to enhance antitumor immune response by using genetically modified tumor cells that had been transfected with CD70 (16,17). As a result, NK cell-mediated tumor rejection was successfully induced, as was tumor-specific T cell memory (16).

CD44 is the principal receptor for transmembrane hyaluronate and preferentially expressed by different cell types. It plays an important role in physiological processes, including cell migration, hemopoiesis and lymphocyte homing (18,19). In addition, CD44 has an important role in the local invasion, growth and metastasis of tumor. In fact, serum concentrations of soluble CD44 are relatively increased in patients with malignant diseases, including colorectal metastases, breast, pancreatic and stomach cancers (20,21). CD44 exists in a variety of isoforms (CD44s, CD44H, CD44v3–6 and v9) generated by alternative splicing of the pre-messenger RNA (mRNA). In addition, an elevated serum level of soluble CD44 and CD44 variants may be useful as a prognostic indicator of tumor burden and metastasis in cancer patients, especially with diffuse type stomach carcinoma (22,23).

IL-18 was recently identified as a new member of the IL-1 cytokine family. It is expressed in many cell types including macrophages, Kupffer cells, dendritic cells and some tumor cells and within the adrenal cortex and pituitary gland (24,25). It is synthesized as a 24 kDa inactive precursor, but this is cleaved by IL-1β-converting enzyme (caspase-1) in the cytoplasm and secreted as an 18 kDa active form, and in this form it participates in interferon-γ production from Tγδ and NK cells (26). For this reason, it was designated as an interferon-γ-inducing factor (27). IL-18 also upregulates Fas ligand expression on NK cells to augment NK cell cytotoxicity (28). In addition, IL-18 contributes to systemic and local inflammation by producing TNF-α, granulocyte-macrophage colony-stimulating factor, inductive nitric oxide synthase and cyclooxygenase-2 (COX-2) (29,30). According to recent reports, IL-18 is not only able to enhance TNF-α responses but can also induce naive CD4+ T cells to differentiate into IL-4-producing Th2 cells in the absence of IL-12 (31). We reported previously that IL-18 is secreted by B16 murine melanoma cell

Abbreviations: Ab, antibody; COX-2, cyclooxygenase-2; ELISA, enzyme-linked immunosorbent assay; HLA, human leukocyte antigen; IL, interleukin; IL-18BP, IL-18-binding protein; MHC, major histocompatibility antigen; mRNA, messenger RNA; NK, natural killer; PBS, phosphate-buffered saline; sCD44, small interfering RNA; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

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line and that it is involved in the immune escape of murine melanoma cells (32). Furthermore, ex vivo studies suggest that enhanced IL-18 expression is positively correlated with the pathogenesis of malignant skin tumors, such as squamous cell carcinoma and melanoma, indicating that it has an important role in the malignancy of skin tumors (33). IL-18 also regulates hepatic melanoma metastasis by increasing the expression of vascular cell adhesion molecule-1 and the adherence of melanoma cells (34).

In the present study, we investigated the biological roles of endogenous IL-18 on immune escape and metastasis of stomach cancer cells via the regulation of CD70, CD44 and vascular endothelial growth factor (VEGF) expression.

Materials and methods

Cells

The human stomach cancer cell line NCI-N87, SNU1, SNU16, SNU601 and SNU638 was obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated fetal bovine serum. This cell line was used for experiments while in the log phase of growth.

Immunohistochemical staining of tumor tissues

Tissues were obtained from 10 patients with stomach cancer who is undergoing operation. The tissues were kindly provided by Dr H.-K.Y., Division of General Surgery in Seoul National University Hospital (Seoul, Korea). According to the pathological findings, all of tumor masses were locally existed, not distantly spread. Immediately after surgery, the tissue samples were snap frozen in OCT Tissue Tek embedding medium (Miles, Elkhart, IN) and stored at −80°C. The frozen stomach cancer tissues were cryosectioned to 5 µm at −20°C. For identification of IL-18, immunohistochemistry of stomach cancer tissues was performed using alkaline phosphatase-anti-alkaline phosphatase method. Briefly, the slides were incubated with polyclonal rabbit anti-human IL-18 antibody (Ab) (1:25) for overnight at room temperature. Slides were washed three times with phosphate-buffered saline (PBS) and then treated with goat anti-rabbit immunoglobulin G (1:100, 1 h). Then, alkaline phosphatase-anti-alkaline phosphatase (1:25, 1 h), respectively. The last two steps were repeated once more. After slides were washed with 0.1 M Tris–Cl (pH 6.8) for 10 min, the color was developed by 30 min incubation with Fast Red (Sigma, St Louis, MO). Stop the color development by washing with tap water and counter staining was done by using methyl green.

IL-18 and VEGF enzyme-linked immunosorbent assay

The concentration of IL-18 in the culture supernatant and sera from stomach cancer patients (n = 20) was measured by enzyme-linked immunosorbent assay (ELISA). Cancer patients' sera were kindly provided by Dr H.-K.Y., the Division of General Surgery in Seoul National University Hospital (Seoul, Korea) and normal control sera were collected from healthy volunteers (n = 20) in Seoul National University College of Medicine. Human IL-18 ELISA kits were purchased from R&D systems (Minneapolis, MN) and ELISA was performed according to the manufacturer's instructions. Briefly, culture supernatants and sera were added to anti-IL-18 Ab-coated well. After 1 h incubation at 37°C, the wells were washed four times with PBS–Tween20 (pH 7.4), alkaline phosphatase-conjugated anti-human IL-18 Ab was added and then incubated for another 1 h. After the wells were washed four times with PBS–TWEEN20, substrate solution was added and incubated for another 1 h. The relative absorbance was measured at 450 nm and then IL-18 concentration was calculated with IL-18 standard curve. The concentration of VEGF levels between IL-18 wild-type and IL-18 small interfering RNA (siRNA)-transfected stomach cancer cells was also performed by ELISA. Human VEGF ELISA kit was purchased from R&D systems and ELISA was performed according to the manufacturer’s instructions. The procedures were the same as the above described.

Reverse transcription–polymerase chain reaction

Total RNA was isolated from 1 × 107 cultured cells using RNAzol, and complementary DNA was made using Superscript II RT ( Gibco BRL, Carlsbad, CA) and oligo (dT)15 (Promega, Madison, WI) as a primer. Then, complementary DNA was amplified with β-actin primers (5′-TAGGCGGGTTACCCCAACACTGTGCCCCATCTA-3′ and 5′-CTAGAACCTTGGCGTGTGA-3′) product = 660 bp) and IL-18 receptor primers (5′-TA- CCTAAAAAGAATGCCGACCTGA-3′ and 5′-TCTTTTGGCGTGTCACTGC- TCT-3′; product = 514 bp). Cycling conditions for IL-18 receptor were 1 min at 95°C, 1 min at 60°C and 30 s at 72°C for 30 cycles. And cycling conditions for β-actin were 30 s at 94°C, 30 s at 56°C and 1 min at 72°C for 23 cycles. Polymerase chain reaction products were electrophoresed and the density of each band was analyzed.

Flow cytometry analysis

The distribution of IL-18 receptor, CD70 and CD44 on human stomach cancer cells line was assessed by flow cytometry using rat anti-human IL-18 Ab (R&D systems), fluorescein isothiocyanate-conjugated mouse anti-human CD70 Ab, phycoerythrin-conjugated mouse anti-human CD44 Ab and anti-mouse-Ig (Pharmingen, San Diego, CA). Cells were washed twice in PBS and then incubated by incubating for 30 min on ice, followed by three washes. An Epics ALTRA (Coulter co., Fullerton, CA) flow cytometer was used for analysis.

Cytotoxicity assay

NK cell and cytotoxic T lymphocytes cytotoxicity was measured in a standard 4 h 51Cr-release assay. Briefly, target cells were prepared through 18 h pre-treatment of IL-18-binding proteins (1 µg) or IL-18-specific siRNA transfection to stomach cells. One million target cells were labeled with 25 µl Na51CrO4 (1 mM; Amersham International, Bucks, UK) by incubation for 1.5–2 h at 37°C in 5% CO2. Labeled cells were washed three times in complete medium and resuspended in complete RPMI 1640 medium at a concentration of 1 × 106 cells/ml. Mixtures of 100 µl of effector cells and 50 µl of 51Cr-labeled target cells (5 × 105 cells) were incubated for 4 h at 37°C in 5% CO2 in 96-well U-bottom culture plates (Nunc, Kamstrup, Denmark) at various ratios of effector and target cells (ranging from 25:1 to 100:1). After centrifugation at 1500 r.p.m. for 5 min, 100 µl of supernatant was harvested from each well, and 51Cr release was determined using a gamma scintillation counter (Packard Instrument Company, Downers Grove, IL). Spontaneous and maximum releases were determined from aliquots of supernatants from which only radiolabeled target cells were incubated in either complete medium or in 2% NP40. Spontaneous lysis ranged from 8 to 10% of the maximum release. The percent specific lysis was calculated by the following formula: percent specific lysis = (experimental 51Cr release–spontaneous 51Cr release/ maximum 51Cr release–spontaneous 51Cr release) × 100.

Preparation and transfection of siRNA

The siRNA sequence targeting IL-18 corresponded to the coding region 445–468 (5′-GATAGGCACCTGATGTTGCTTG-3′) relative to the start codon. The siRNA duplex with the following sense and antisense sequences was used: 5′-GAUAGCGACCUAGAGGAUdTdT-3′ (sense) and 3′-dTdT- CUAUGGGUGGAUCUCUAU-5′ (antisense). The control siRNA duplex has the following sequences: 5′-AGGGAGACUAUAGGUGACG-3′ (sense) and 3′-dTdT-UCCUAAUUGUACGACG-5′ (antisense). All of the siRNA duplexes were labeled with fluorescein after synthesis by Proligo Singapore Pte Ltd (Singapore, Singapore). Cells in exponential phase of growth were plated in six-well plates at 5 × 105 cells/well, grown for 24 h and then transfected with 20 nM of siRNA using oligofectamine and OPTI-MEM I-reduced serum medium (Invitrogen Life Technologies, Carlsbad, CA), according to the manufacturer’s protocol. The concentrations of siRNA were chosen based on dose-response studies. After examination of transfection efficacy under fluorescent microscopy, silencing was examined by intracellular flow cytometry at 24–48 h after transfection. Control cells were transfected with control siRNA with oligofectamine and serum-reduced medium (mock).

Intracellular flow cytometry analysis

Intracellular fluorescence activated cell sorter analysis was performed to detect the production of IL-18 in siRNA-transfected stomach cancer cell lines. Briefly, cells were washed twice with ice-cold PBS containing 0.05% bovine serum albumin and 0.02% sodium azide. After two washes, cells were fixed in 2% paraformaldehyde in PBS for 15 min on ice. Thereafter, the cells were washed once in cold PBS–bovine serum albumin and resuspended in PBS containing 0.1% saponin and 0.05% sodium azide (permeabilization buffer) for 15 min, followed by incubation with mouse anti-human IL-18 polyclonal antibody (Pharmingen) for 30 min on ice. After two washes, cells were further incubated with an appropriate fluorescein isothiocyanate-conjugated secondary antibody in permeabilization buffer for 30 min on ice, followed by three washes. An Epics ALTRA (Coulter co.) flow cytometer was used for analysis.

Animal study

Bab/C (nu/nu) male mice 6–8 weeks of age were obtained from the Jackson Laboratory (Bar Harbor, ME). The human stomach tumors were established in Bab/C (nu/nu) mouse by subcutaneous injection of wild-type and IL-18 siRNA-transfected NCI-N87 stomach cancer cells (1 × 105) in 200 µl of PBS. Tumor size was determined by measuring with calipers every 3–4 days, and the values were inserted into the formula: Tumor volume (mm3) = 0.5 × (largest diameter) × (smallest diameter)2.
IL-18 expression in tumor tissues from stomach cancer patients. (A) The sera from stomach cancer patients (n = 20) and healthy normal individuals (n = 20) were diluted (1:10) with serum dilution buffer and then performed IL-18 ELISA as described in Materials and Methods. Results are representative of three experiments, each performed in triplicate. *P-value of stomach cancer patients was <0.05, when it compared with healthy normal individuals (P-value = 0.0000000973). (B) Tissues were obtained from 10 patients with stomach cancer who is undergoing operation. Immediately after surgery, tissues are frozen and cryosectioned for detection of IL-18 expression on stomach cancer tissues by alkaline phosphatase anti-alkaline phosphatase method as described in Materials and Methods. Results are representative of 10 experiments, each performed in duplicate.
Generation of IL-18 siRNA-transfected stomach cancer cells by siRNA transfection

To confirm the results in neutralizing experiments from above and to perform further in vivo experiments, we tried to generate IL-18 siRNA-transfected stomach cancer cells by using siRNA that specifically block the expression of IL-18 mRNA. Since we have used fluorescein labeled IL-18 siRNA in this experiment, we confirmed transfected IL-18 siRNA inside of the stomach cancer cells under the fluorescent microscopy (data not shown). And then, we determined the efficiency of IL-18 siRNA transfection into stomach cancer cells by measuring IL-18 production 2 days after transfection. As shown in Figure 4A and B, decrease of IL-18 production was only shown in IL-18 siRNA-transfected NCI-N87 stomach cancer cells but not in IL-18 siRNA-transfected SNU16 stomach cancer cells. Even though we did the transfection...
experiments > 10 times, SNU16 stomach cancer cells always showed marginal transfection efficiency and we failed to make an IL-18 siRNA-transfected SNU16 stomach cancer cell. Therefore, we performed further experiments only using IL-18 siRNA-transfected NCI-N87 stomach cancer cells.

Increase of CD70 expression and immune susceptibility of IL-18 siRNA-transfected NCI-N87 stomach cancer cells

We have already shown that CD70 expression and immune susceptibility of stomach cancer cells were increased after neutralization of endogenous IL-18 by IL-18BP. Therefore, we examined the changes of CD70 expression and immune susceptibility of IL-18 siRNA-transfected NCI-N87 cells. In accordance with the result using IL-18BP, we observed the increased CD70 expression on IL-18 siRNA-transfected NCI-N87 cells (Figure 4C) and increased immune susceptibility of IL-18 siRNA-transfected NCI-N87 cells (Figure 4D).

The suppression of the phosphorylation of Akt by endogenous IL-18 from tumor

To examine the signaling mechanism of IL-18 to suppress CD70 expression and maintain CD44 expression, we blocked the interaction of IL-18 and its receptor on stomach cancer cell line by the IL-18BP. After blocking of the interaction between IL-18 and IL-18R for 24 h, the changes on the phosphorylation of several signaling molecules were subjected. As shown in Figure 5, there were no remarkable changes on extracellular signal-regulated kinase and p38MAPK by the inhibition of the interaction of IL-18 and its receptor, but the phosphorylation on Akt was dramatically increased. In the case of c-jun N-terminal kinase, it was also slightly increased. Therefore, it seems that the phosphorylation of Akt is achieved by the binding of IL-18 to its receptors on the surface of stomach cancer and its suppression is closely related with the immune escape and metastasis of stomach cancer via the suppression of CD70 expression and maintenance of CD44.

Decrease of CD44 and VEGF expression on stomach cancer by silencing and neutralization of endogenous IL-18

It is already reported that IL-18 plays an important role not only in angiogenic process but also in the metastasis of tumors, such as lung cancer, breast cancer and melanoma (34–37). In addition, CD44 has a critical role in the preferential metastasis of stomach cancer to the peritoneum. Therefore, we examined the changes of CD44 expression by the modulation of endogenous IL-18 production from stomach cancer. As a result, CD44 expression was decreased in both stomach cancer cells (SNU16 and NCI-N87) after neutralizing endogenous IL-18 with IL-18-binding protein (Figure 6A and B). We also confirmed the decrease of CD44 expression on IL-18 siRNA-transfected NCI-N87 stomach cancer cells (Figure 6C). It implies that stomach cancer cells maintain the high expression of CD44 by using endogenous IL-18 for their metastasis to other sites. In relation to the angiogenic process by tumor, VEGF is widely recognized as a potent angiogenic factor. Therefore, we examined whether endogenous IL-18 also affects the production of VEGF from NCI-N87. As shown in Figure 6D, we found the decreased VEGF production on IL-18 siRNA-transfected NCI-N87. And we gained the same results when the endogenous IL-18 from NCI-N87 is neutralized with IL-18BP (data not shown). These data show that stomach cancer cells use IL-18 to increase CD44 expression and VEGF production.

Cox-2 affects VEGF production induced by IL-18

It is known that IL-18 induces the production of inducible nitric oxide synthase and Cox-2, when it contributes to systemic and local inflammation. In addition, decreased expression of Cox-2 was observed in NCI-N87, when they were incubated in the presence of IL-18BP (Figure 7A). Therefore, we examined whether Cox-2 acts as...
Fig. 5. The suppression of the phosphorylation of Akt by endogenous IL-18 from tumor. The changes on signaling molecules by endogenous IL-18 from stomach cancer cells were investigated by immunoblotting as described in Materials and methods. Briefly, cells were cultured in the presence of IL-18-binding proteins for 24 h and then lysed in ice-cold lysis buffer. Thirty micrograms of each sample were loaded into 12% polyacrylamide gels, electrophoresed and transferred to nitrocellulose membranes. Membranes were blotted with mouse developed anti-human Akt/p-Akt/p38MAPK/ pp38MAPK/JNK/p-JNK Ab and c-jun N-terminal kinase (JNK).

a mediator in IL-18-induced VEGF production. For this, we made Cox-2 antisense cell stomach cancer cell line using Cox-2-specific siRNA and the silencing effect of Cox-2 siRNA was examined by immunoblotting at 48 h after transfection (Figure 7B). When NCI-N87 was incubated with 20 and 40 μM NS-398, VEGF expression was decreased in a dose-dependent manner (Figure 7C). In addition, decrease of CD44 expression was also showed on Cox-2 antisense stomach cancer cells (Figure 7C). Therefore, it seems that Cox-2 plays important role on the regulation of VEGF production by IL-18.

The tumorigenecity and metastasis of IL-18 siRNA-transfected stomach cancer cells in vivo
As shown in results from our in vitro experiment using IL-18BP and IL-18 siRNA-transfected NCI-N87 stomach cancer cells, endogenous IL-18 from stomach cancer plays an important role in immune escape and metastasis of stomach cancer. To investigate whether the deletion of endogenous IL-18 also elicits an effective antitumor immune response in vivo, we established human stomach tumors in the Balb/C (nu/nu) mouse by injecting control siRNA and IL-18 siRNA-transfected NCI-N87 stomach cancer cells into subcutaneous region. Tumor sizes were determined by measuring with calipers every 3–4 days for 6 weeks after the inoculation. Size and weight of tumors, in Balb/C (nu/nu) mice injected with an IL-18 siRNA-transfected NCI-N87 stomach cancer cells, were smaller than those in control siRNA-transfected stomach cancer cell line-injected Balb/C (nu/nu) mice (Figure 8A and B). Therefore, it appears that effective antitumor immune response can be elicited in vivo by the deletion of endogenous IL-18 and tumor rejection can be achieved by NK cells.

The decreasing CD44 expression and VEGF production on IL-18 siRNA-transfected NCI-N87 stomach cancer cells were already shown in Figure 6C and D. Therefore, we investigated the effects of decreased CD44 expression and VEGF production on the metastatic ability of stomach cancer cells in stomach tumor-established Balb/C (nu/nu) mice (n = 6). After 6 weeks of tumor inoculation, mice were killed and lung specimen was removed and then subjected to hematoxylin and eosin staining. As shown in Figure 8C–E, two metastatic foci were observed in lung specimen from control siRNA-transfected NCI-N87 stomach cancer cells-inoculated mice (n = 6). However, there were no metastatic regions on IL-18 siRNA-transfected NCI-N87 stomach cancer cells-inoculated mice (n = 6) (Figure 8F).

Interestingly, we also found the inhibition of neovascularization near tumor mass in IL-18 siRNA-transfected NCI-N87 stomach cancer cells-injected mice, when it compared with the control mice injected from control siRNA-transfected NCI-N87 stomach cancer cells (Figure 8G and H). Taken together, stomach cancer cells produce IL-18 for their metastasis via the sustenance of CD44 expression and for the angiogenic process via the production of VEGF.

Discussion
Stomach cancer is one of the most frequently diagnosed malignancies worldwide, especially in East Asian countries including Korea (1). Several factors including molecular and genetic alterations and infection with *H.pylori* are considered to be associated with the development of stomach cancer. Molecular and genetic alterations, such as the inactivation of p53, and changes in oncogene (ras, c-met and c-erbB2) expression are typically observed in stomach cancer patients and cause a poor prognosis (38,39). The factor most strongly associated with the development of stomach cancer is *H.pylori* infection (2,3), which induces the secretion of inflammatory cytokines including IL-18, IL-8, IL-10 and interferon-γ and increases oxidative stress (40,41).

A recent report concerned the relationship between infection with *H.pylori* and a new member of the inflammatory cytokine, IL-18. Fera et al. (3) reported that active IL-18 production is increased in patients with chronic gastritis associated with *H.pylori* infection. In addition, it was reported that the mean serum IL-18 level in stomach cancer patients who underwent curative surgery was significantly higher compared than that of normal individuals (37), which implies that IL-18 can be used as a marker of stomach cancer prognosis, but does not explain its function on cancer cells and the immune system. Therefore, we examined its effects on the pathogenesis of stomach cancer.

Cancer cells are known to use several mechanisms, including the downregulation of major histocompatibility antigen (MHC), costimulatory molecules and Fas expression to escape from the immune system and to metastasize rapidly (42–44). We postulated that cancer cells might use endogenous products, such as cytokines, especially IL-18 for immune escape. Thus, we screened for possible costimulatory molecules that are changed by IL-18 modulation. Among the many costimulatory molecules, we focused on the upregulation of CD70, which is able to increase the cytotoxicity of NK cells and induce tumor-specific T cell memory in stomach cancer cells (15). The expression of CD70 is increased by the neutralization or deletion of IL-18 and this seems to result in an increase in the susceptibility of tumor cells to NK cells or cytolytic T lymphocytes, but this effect was not as dramatic as expected. Therefore, we also investigated whether Fas (CD95), MHC class I and HLA-G are involved in this process since they are known cytotoxicity-related molecules. We were unable to detect any changes in the expressions of Fas or MHC class I after IL-18 neutralization which is known to deliver inhibitory signal that counteracts the activation signal of NK cells and to prevent the destruction of target cells by NK cells (45,46), increased slightly. Based on those results, it seems that the increased immune susceptibility of stomach cancer cells after IL-18 neutralization is caused by the upregulation of
 However, several studies have reported on the synergistic costimulatory effect of CD70 and CD80 (17). Therefore, we do not exclude the cooperative effects of other costimulatory molecules (except Fas, MHC class I and HLA-G) with CD70.

CD44 is the most well-known molecule that implicated in the preferential metastasis of stomach cancer to the peritoneum. In addition, Jiang et al. (47) reported that IL-18 might play a role in metastasis by inhibiting E-cadherin expression. We investigated the effect of IL-18 on metastasis of stomach cancer cell lines.

Fig. 6. Decrease of CD44 and VEGF expression in stomach cancer cell lines by modulation of endogenous IL-18 expression (A and B). Human stomach cancer cell lines, NCI-N87 (A, left) and SNU16 (B, right), were neutralized by 1 μg of IL-18-binding protein for 18 h and analyzed CD44 expression by flow cytometry as described in Materials and Methods. Results are representative of more than three experiments. (C) CD44 expression on IL-18 siRNA-transfected NCI-N87 stomach cancer cells were assessed by flow cytometry. Results are representative of three experiments. (D) Control siRNA or IL-18 siRNA-transfected NCI-N87 stomach cancer cells (5 x 10⁶) were cultured for 24 h and culture supernatants were collected. And then VEGF ELISA was performed as described in Materials and Methods. Results are representative of three experiments, each performed in triplicate. *P-Value of IL-18 siRNA-transfected NCI-N87 was <0.05, when it compared with control siRNA-transfected NCI-N87.

Fig. 7. Cox-2 affects VEGF production induced by IL-18. (A) NCI-N87 was incubated in the presence or absence of IL-18-binding protein (IL-18BP) for 6 h and then change in Cox-2 expression was examined by flow cytometry. Results are representative of three experiments. (B) After transfection of 20 μM of siRNA targeted to Cox-2 mRNA, and the silencing efficiency was investigated by immunoblotting. Results are representative of three experiments. (C) After culture of NCI-N87 with or without NS-398 and Cox-2 antisense NCI-N87 for 24 h, culture supernatants were collected. And then change in VEGF production was examined by VEGF ELISA. Results are representative of more than three experiments, each performed in triplicate. *P-Value of Cox-2 siRNA-transfected NCI-N87 and NS-398-treated NCI-N87 were <0.05, when it compared with control.
endogenous IL-18 on the expression of the metastasis-related molecule, CD44. As we expected, its expression was inhibited by the neutralization of IL-18. Therefore, endogenous IL-18 was found to modulate the metastasis of stomach cancer cells by maintaining high CD44 expression. The CD44 has extensive size heterogeneity, ranging from 85–95 kDa form to larger variant of 200 kDa or more (22). In the present experiment, we investigated the changes of CD44 expression by flow cytometry only. Therefore, the analysis of expression pattern of

Fig. 8. The effect of IL-18 on growth, angiogenesis and metastasis of stomach cancer in vivo. (A) Tumors were established in the Balb/C (nu/nu) mice (n = 6) by subcutaneous injection of $1 \times 10^7$ of control siRNA or IL-18 siRNA-transfected stomach cancer cells in 200 µl of PBS. And then tumor size was determined by measuring with calipers every 3–4 days, and the values were inserted into the formula: Tumor volume (mm$^3$) = $0.5 \times$ (largest diameter) $\times$ (smallest diameter)$^2$. $^*P < 0.05$ compared with corresponding values for control siRNA transfectant-injected mice. (B) The mean weight of tumor mass; Mice were killed at 6 weeks after tumor inoculation and then the weight of tumor mass was measured. Data represents mean ± SD. $^*P < 0.05$ compared with corresponding values for control siRNA transfectant-injected mice. (C–F) Balb/C (nu/nu) mice were killed at 4 and 6 weeks after injection of $1 \times 10^7$ of control siRNA-transfected stomach cancer cells (C and E) or IL-18 siRNA-transfected stomach cancer cells (D and F) and then examine the formation of newly synthesized blood vessels near tumor mass. (G–J) Lungs were removed from killed mice and frozen rapidly. And then cryosectioned and stained in eosin. By light microscopy, the number and location of metastatic lesions were assessed. (G) Lung from control siRNA-transfected NCI-N87 stomach cancer cells injected mice, $\times$100, (H) magnified metastatic lesion (a) to $\times$400, (I) magnified metastatic lesion (b) to $\times$400 and (J) lung from IL-18 siRNA-transfected NCI-N87 stomach cancer cells-injected mice, $\times$100 (K) Spleens from control siRNA transfectant and IL-18 siRNA transfectant-injected mice.
CD44 variant in stomach cancer cells by endogenous IL-18 should be further investigated. According to the reports by Dohadwala et al. (48), COX-2 has a critical role in the regulation of CD44-dependent invasion by human non-small cell lung cancer. In addition, it is known that IL-18 induces the production of inducible nitric oxide synthase and COX-2, when it contributes to systemic and local inflammation. We observed that expression of COX-2 mRNA transcripts was decreased in NCI-N87, when they were incubated in the presence of IL-18BP (Figure 7A).

We designed a siRNA against IL-18 and used it to make an IL-18 (−/−) stomach cancer cell line by transfection into the stomach cancer cells line NCI-N87 and SNU16. Transfection efficiencies were determined by measuring IL-18 production by intracellular flow cytometry and ELISA. IL-18 siRNA used in our experiments was not properly act in SNU16 because the functional activities of siRNA differ according to their mRNA recognition sequences in the different host cells (49). Accordingly, control siRNA and IL-18 siRNA-transfected NCI-N87 stomach cancer cells were established in Balb/C (nu/nu). As shown in Figure 4A, the relatively smaller tumors occurred in IL-18 siRNA-transfected NCI-N87 stomach cancer cells than in control siRNA-transfected NCI-N87. Together with the results of our in vitro study, this strongly suggests that effective tumor rejection can be achieved by blocking endogenous IL-18 production by upregulating CD70. However, tumor sizes in two groups showed parallel increases from 21 days after inoculation. In general, it is known that only transient transfectants can be made using siRNA and the effect of transfected siRNA commonly diminishes 9–10 generations after transfection. IL-18 siRNA-transfected NCI-N87 stomach cancer cells were inoculated at the five to six generation after IL-18 siRNA transfection. Therefore, it is because of the declined effect of IL-18 siRNA that parallel increases of tumor sizes in two groups at 21 days after tumor inoculation.

To determine the correlation between decrease of CD44 on IL-18 siRNA-transfected NCI-N87 stomach cancer cells and their metastatic ability, mice were killed at 6 weeks after tumor inoculation. And then their spleen, lung and liver were compared with those of the control siRNA-transfected NCI-N87-injected mice. It is known that sphenomegalay is a typical characteristic of tumor-bearing mice. Control siRNA-transfected stomach cancer cell line-injected Balb/C (nu/nu) mice had an enlarged spleen, but spleens in mice, bearing the tumor originating from IL-18 siRNA-transfected NCI-N87 stomach cancer cells, were the same as that of the PBS control, even though all animals had a tumor mass in the subcutaneous region (Figure 8K). In addition, we were unable to find metastatic tumors in liver or lungs of IL-18 siRNA transfectant-injected mice, whereas several metastatic regions were observed on lung specimens from IL-18 wild-type. In accordance with this result, the neovascularization was predominantly occurred near the tumor mass originating from control siRNA-transfected NCI-N87 cells but did not occur near the tumor mass originated from IL-18 siRNA-transfected NCI-N87 cells (Figure 8C–F).

To our knowledge, our present study is the first one regarding the role of endogenous IL-18 on the immune escape mechanism and metastasis of stomach cancer. When we get together with results from in vitro and in vivo study, it strongly suggests that the effective tumor rejection and suppression of metastasis can be achieved by blocking of endogenous IL-18 production through the upregulation of CD70 expression and downregulation of CD44 expression and VEGF production, respectively. Therefore, we assure that our data will provide the new insights for the therapeutic methods of stomach cancer.

Funding

Korean Science and Engineering Foundation, Tumor Immunity Medical Research Center, Seoul National University College of Medicine (R13-2002-025-02001-0); Science Research Center program, Research Center for Women’s Diseases (R11-2005-017-03001).

Acknowledgements

Conflict of Interest Statement: None declared.

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Received November 11, 2008; revised June 18, 2009; accepted June 18, 2009