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IL-18 enhances thrombospondin-1 production in human gastric cancer via JNK pathway ☆,☆☆

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Abstract

IL-18 is a pleiotropic cytokine that is produced by many cancer cells. A recent report suggested that IL-18 plays a key role in regulating the immune escape of melanoma and gastric cancer cells. Thrombospondin (TSP-1) is known to inhibit angiogenesis in several cancers but some studies have reported that it stimulates angiogenesis in some cancers such as gastric cancer. IL-18 and TSP-1 are related to tumor proliferation and metastasis. This study investigated the relationship between IL-18 and TSP-1 in gastric cancer. RT-PCR and ELISA showed that after the cells had been treated with IL-18, the level of TSP-1 mRNA expression and TSP-1 protein production by IL-18 increased in both a dose- and time-dependent manner. The cells were next treated with specific inhibitors in order to determine the signal pathway involved in IL-18-enhanced TSP-1 production. IL-18-enhanced TSP-1 expression was blocked by SP600125, a c-Jun N-terminal kinase (JNK) specific inhibitor. In addition, Western blot showed that IL-18 enhanced the expression of phosphorylated JNK. Overall, these results suggest that IL-18 plays a key role in TSP-1 expression involving JNK.

Keywords: IL-18; TSP-1; Gastric cancer; JNK pathway

IL-18 is a member of the IL-1 family of ligands. The term was formerly used to describe the IFN-γ inducing factor [1,2]. IL-18 is produced as a 24 kDa inactive precursor (pro-IL-18), which lacks a signal peptide. Pro-IL-18 is converted by caspase-1 to 18 kDa, which is in a biologically active form [3,4]. This active IL-18 form can be secreted by LPS-activated macrophages, keratinocytes as well as some tumor cells [2,5]. The reported effects of IL-18 on the immune system include the enhancement of NK cell activity and the proliferation of activated T cells [6]. IL-18 stimulates the Th1 cytokines, including IFN-γ by T and NK cells, and promotes Th1 cell differentiation and immune responses. IL-18 may also facilitate the development of the Th2 responses. Therefore, IL-18 is a unique cytokine that enhances both the Th1- and Th2-derived immune responses [1]. Recently, IL-18 was associated with the immune escape of tumor cells, such as melanoma [7] and gastric cancer [8], and acted as a growth factor for murine melanoma cells [9]. In addition, IL-18 was reported to increase the invasiveness of myeloid leukemia cells [10]. Gastric cancer patients with elevated serum IL-18 levels were shown to have a significantly unfavorable outcome and more rapid tumor progression [11,12].

Angiogenesis is a key step in the proliferation and metastasis of cancer. Solid tumors, <1 to 2 cubic

☆ *Abbreviations:* IL-18, interleukin-18; TSP-1, thrombospondin-1; JN, c-Jun N-terminal kinase.
☆☆ Basic finding of this paper: IL-18-enhanced TSP-1 production plays an important role in the progression and pathogenesis of gastric cancer cells.
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millimeters, are not vascularized. In order for them to spread, they must be supplied by blood vessels that bring oxygen and nutrients, and remove metabolic waste. VEGF, as well as PDGF, TSP-1, and FGF, plays an important role in the growth and stabilization of these new vessels. However, TSP-1, angiostatin, and vasostatin also have an anti-angiogenesis role. As mentioned above, TSP-1 has a controversial role in angiogenesis and tumor progression. TSP-1 is a 450 kDa multifunctional glycoprotein that is mainly stored in the α-granules of platelets [13]. TSP-1 is also secreted by a variety of cells including endothelial cells, macrophages, fibroblasts, and smooth muscle cells [14], and binds to the extracellular matrix [15]. TSP-1 plays an important role in wound healing and activates the latent TGF-β_1 complex via a protease- and cell-independent mechanism in vitro [16]. Thrombospondins have five members, TSP-1 to TSP-5. TSP members are multi-meric as well as multi-modular heparin and calcium-binding proteins. The TSPs can be divided into two subfamilies. TSP-1 and TSP-2 have a similar structural organization. Each subunit of these trimeric molecules is composed of an N-terminal heparin-binding domain, a procollagen-homology domain, three type I repeats, three epidermal growth factor (EGF)-like type II repeats, seven calcium-binding type III repeats, and a C-terminal globular domain. The other TSP proteins, TSP-3, TSP-4, and TSP-5/COMP, resemble one another but are pentameric, and that their subunits possess four type II repeats instead of three. In addition, they lack the procollagen-homology domain and the three type I repeats. TSP-1 and TSP-2 have been the most investigated of the TSP families. TSP-1 plays a role in tumor progression [17].

IL-18 and TSP-1 are relevant to tumor proliferation and metastasis. However, the relationship between IL-18 and TSP-1 is unclear in cancer. Therefore, this study examined the affect of IL-18 on TSP-1 expression in the gastric cancer cell line, SNU-601.

Materials and methods

Reagents. The recombinant human IL-18 was purchased from MBL (Nagoya, Japan). The SP600125 (a JNK inhibitor II), PD98059 (an ERK1/2 inhibitor), H-89 (a protein kinase A inhibitor), and Bisindolylmaleimide I (a protein kinase C inhibitor) were obtained from Calbiochem (San Diego, CA). The rabbit (polyclonal) anti-JNK 1 and 2 [pTpY$^{183/185}$] phosphospecific antibody and rabbit (polyclonal) anti-JNK 1 pan antibody are supplied by Biosource.

Cell culture. The human gastric cancer cell line, SNU-601, as well as the SNU-1 and MKN-28 cell lines, was obtained from KCLB (Korean Cell Line Bank of the Cancer Research Center at Seoul National University's college of Medicine). The cells were cultured in complete medium (CM) (RPMI1640 medium (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamin, 100 U/ml penicillin, and 100 g/ml streptomycin) or serum-free media (without the 10% heat-inactivated fetal bovine serum). The cells were maintained in a 5% CO_2 incubator at 37 °C and used for the experiments whilst in the log phase.

Flow cytometry analysis. Indirect flow cytometry analysis was used to detect the IL-18 receptor on the SNU-601 cells. Aliquots of the cells were placed into FACS tubes, washed twice in phosphate-buffered saline (PBS), and labeled with 10 μg/ml of mouse anti-human IL-18 receptor Ab (R&D systems, Minneapolis, MV) for 30 min on ice. The cells were then washed three times, and incubated with the secondary Ab, FITC-conjugated goat anti-mouse IgG (1:100) (Pharmingen, San Diego, CA), for 30 min on ice. The cells were then washed twice in PBS and the labeled cells were measured by flow cytometry.

Reverse transcriptase-polymerase chain reaction (RT-PCR). The total RNA was extracted from 1×10^7 cells using easy-blue (Intron, kyunggi-korea). The RNA purity and quantity were determined using Nano drop. cDNA was then made from 5 μg of the total RNA using a cDNA synthesis kit (Intron). The cDNA was used for PCR analysis with the human β-actin primers (5′-TAG CGG GGT TCA CCC ACA CTG TGC CCC ATC TA-3′/5′-CTA GAA GCA TTT GCG GTG GAC CGA TGG AGG G-3′; target size : 661 bp) or the human TSP-1 primers (5′-CCG ACC AGA AGG ACT CTG AC-3′/5′-GGC TGA GAC GCC ATC GAG AG-3′; target size : 594 bp). There were 30 cycles for β-actin consisting of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min. There were 35 cycles for TSP-1 consisting of 94 °C for 1 min, 58 °C for 45 s, and 72 °C for 2 min, which was followed by denaturation for 5 min at 94 °C (β-actin : 95 °C). After the final cycle, an extension step was carried out at 72 °C for 5 min. The PCR products were electrophoresed.

Enzyme-linked immunosorbent assay (ELISA). The level of TSP-1 secreted into the culture medium by cells was determined using a competitive ELISA (Chemicon, USA and Canada). The cells were plated at 5×10^6 cells in a 25 flask and incubated for 2–3 days until they were subconfluent. The culture supernatants were harvested and dispensed into plates, and the rabbit anti-human TSP-1 polyclonal Ab was added. After incubation for 3 h at RT, the TSP-1 conjugate was added and the mixture was incubated for 30 min at RT. These wells were washed five times, and streptavidin-alkaline phosphate was added and the wells were incubated for 30 min at RT. After washing five times, a color reagent solution was added and the wells were incubated for 20 min at RT. The color development was quenched using a stop solution. The absorbance of each well at 490 nm was measured using an ELISA reader (Molecular Devices, Sunnyvale, CA, USA). The TSP-1 concentration was determined from a standard curve.

Result

Effect of IL-18 on TSP-1 mRNA and protein production in SNU-601

The effect of IL-18 on TSP-1 was analyzed by confirming IL-18R expression on SNU-601 cells using flow cytometry analysis (Fig. 1). The change in the TSP-1 mRNA expression level on the SNU-601 cells as a result of the IL-18 treatment was determined using RT-PCR. IL-18 increased the TSP-1 mRNA expression level (Fig. 2). The production of the TSP-1 protein was also detected using a time-kinetic and dose titration by ELISA. For the time-course study, the cells were exposed to IL-18 (100 ng/ml) and incubated for 12, 24, and 36 h. The level of IL-18-enhanced TSP-1 secretion increased significantly after 24 h stimulation (Fig. 3A). The production of the TSP-1 protein at various IL-18 doses (25, 50, 75, 100, and 125 ng/ml recombinant human IL-18) was next examined. Fig. 3B shows that 100 ng/ml IL-18 was sufficient to enhance the production of TSP-1 in SNU-601 cells. Incubation with 100 ng/ml IL-18 for 24 h was found to be the optimal condition for TSP-1 production in SNU-601 cells. Overall, these results show that IL-18 increases the production of TSP-1 in both a time- and dose-dependent manner.