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Heterogeneous sphingosine-1-phosphate lyase gene expression and its regulatory mechanism in human lung cancer cell lines

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ABSTRACT

The role of sphingolipid metabolic pathway has been recognized in determining cellular fate. Although sphingolipid degradation has been extensively studied, gene expression of human sphingosine 1-phosphate lyase (SPL) catalyzing sphingosine 1-phosphate (S1P) remains to be determined. Among 5 human lung cancer cell lines examined, SPL protein levels paralleled the respective mRNA and enzyme activities. Between H1155 and H1299 cells used for further experiments, higher cellular S1P was observed in H1155 with higher SPL activity compared with H1299 with low SPL activity. GATA-4 has been reported to affect *SPL* transcription in *Dictyostelium discoideum*. GATA-4 was observed in H1155 but not in other cell lines. Overexpression of GATA-4 in H1299 increased *SPL* expression. However, promoter analysis of human *SPL* revealed that the most important region was located between −136 bp and −88 bp from the first exon, where 2 Sp1 sites exist but no GATA site. DNA pull-down assay of H1155 showed increased DNA binding of Sp1 and GATA-4 within this promoter region compared with H1299. Electrophoresis mobility shift assay (EMSA), chromatin immunoprecipitation (ChIP) assay, reporter assay using mutated binding motif, and mithramycin A, a specific Sp1 inhibitor, suggest the major role of Sp1 in *SPL* transcription and no direct binding of GATA-4 with this 5′ promoter region. The collaborative role of GATA-4 was proved by showing coimmunoprecipitation of Sp1 and GATA-4 using GST-Sp1 and overexpressed GATA-4. Thus, high *SPL* transcription of H1155 cells was regulated by Sp1 and GATA-4/Sp1 complex formation, both of which bind to Sp1 sites of the 5′-SPL promoter.

1. Introduction

Sphingosine 1-phosphate (S1P) is a bioactive lipid produced by sphingosine kinase (SPHK). It promotes cell survival, proliferation and migration, and is also essential for angiogenesis and lymphocyte trafficking [1,2]. Spiegel et al. [3] proposed the sphingolipid rheostat model, where cellular S1P/ceramide and sphingosine ratio determines cell fate. Furthermore, S1P reportedly activates a family of G-protein coupled receptors (GPCR) called $S1P_1$–$S1P_5$, and S1P receptors initiate the cellular signaling pathway after S1P stimulation [4]. Secretion of S1P was also observed in several cells or cell lines [5,6] Thus, it acts as an autocrine or paracrine signal transducer. Based on these results, the sphingolipid rheostat model was modified and the inside-out signaling model of S1P has recently been suggested [7].

Because of the importance of S1P, enzymes that determine the S1P level have recently been intensively studied [4,7]. Two enzymes, SPHK that produces S1P [8,9], and S1P phosphatase that dephosphorylates S1P [10,11], are such examples. In contrast, the regulatory mechanism of human sphingosine 1-phosphate lyase (SPL) that degrades S1P irreversibly has not been well understood. SPL acts as a gate-keeper that regulates S1P signaling by modulating intracellular S1P levels and

Abbreviations: SPL, sphingosine 1-phosphate lyase; DA, daunorubicin; ETP, etoposide; S1P, sphingosine 1-phosphate; Sp1, specificity protein 1; ChIP, chromatin immunoprecipitation; EMSA, electrophoresis mobility shift assay

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the chemical S1P gradient that exists between lymphoid organs and circulating blood and lymph [12,13].

SPL activity has been reported to be located in the endoplasmic reticulum (ER) and human SPL gene encodes a protein of 568 amino acids with a molecular weight of 63.5 kDa [14,15]. Although SPL expression has been observed in most mammalian tissues, its expression level is quite variable; the highest levels were in the liver, kidney and intestine, with lesser expression in muscle and brain [14]. The potential roles of SPL have been reported recently. For example, SPL overexpression as well as sphingosine kinase inhibitor showed the enhanced sensitivity of *Dictyostelium discoideum* cells to the anti-cancer drug, cisplatin [16]. Overexpression of SPL in HEK-293 cells resulted in increased stress-induced apoptosis [17].

However, precise elucidation of the human 5′-promoter of SPL gene and the search for the responsible transcription factors of human cells remain to be determined, especially in cancer cells. Initially, the involvement of GATA transcription factors in the regulatory mechanism of *SPL* gene expression was reported in nematodes [18], and investigations utilizing comparative genomics approaches revealed early growth response factor-, zinc-binding protein factor- and GC-box factor-related motifs in the 5′ untranslated region of the *SPL* gene as the regulatory mechanism of SPL gene expression [19,20]. These reports have also predicted the pathophysiological importance of SPL in human diseases and that the modulation of SPL can be a probable therapeutic approach to cancer and other diseases.

In the current study, we attempted to elucidate the mechanism of human *SPL* transcription by analyzing 2 representative human lung cancer cell lines, H1155 and H1299, that are different in their SPL expression. We showed that Sp1 protein and Sp1 site of the human *SPL* 5′-promoter are important for the basic transcription activity of these cell lines, which was consistent with previous reports of comparative genomics [19,20]. However, Sp1 cannot explain all of the differences of SPL transcription observed in these two cell lines. Therefore, we examined the involvement of GATA-family proteins with SPL transcription as possible explanation. Actually, overexpression of GATA-4 in H1299, a cell line with low SPL expression, increased *SPL* gene expression. Intriguingly, the distal 5′ promoter of human *SPL* lacks consensus GATA binding motifs, suggesting that the direct binding of GATA family to the *SPL* 5′-promoter is less likely. We tried to elucidate the relationship between Sp1 and GATA-4 in SPL transcriptional regulation. Here, we present data showing the interaction of Sp1 and GATA-4 in *SPL* transcription using DNA pull-down assay, chromatin immunoprecipitation (ChIP) assay, electrophoresis mobility shift assay (EMSA), and coimmunoprecipitation experiments using partially purified respective proteins. Our present data reveal the complex regulatory mechanism of SPL transcription in human lung cancer cell lines, which might be one reason for their variable SPL gene expression.

2. Materials and methods

2.1. Cell culture and reagents

Human lung cancer cell lines (H157, H1299, H2347, H460 and H1155) were obtained from Dr. Y. Sekido (Aichi Cancer Center, Nagoya, Japan) and were cultured in 10% fetal calf serum (FCS) in RPMI 1640 medium. Daunorubicin (DA) and etoposide (ETP) were purchased from Sigma (St. Louis, MO, USA). Mouse SPL expression vector, pcDNA3FLAG.mSPL, was the generous gift of Dr. A. Kihara (Hokkaido University, Sapporo, Japan) [21]. GST-Sp1 expression vector was the gift of Dr. JM Horowitz [22] (North Carolina State University, NC, USA). GATA-4 expression vector, pcDNA-GATA-4, was from Dr. Walter L. Miller [23] (University of California San Francisco, CA, USA). Purified GATA-4 was purchased from OriGene Technologies Inc. (Rockville, MD, USA) and was used in some EMSA experiments.

2.2. Real-time RT-PCR

Total RNA was extracted using RNeasy mini kit (QIAGEN, Hilden, Germany). The first strand cDNA was prepared with 2 μg of RNA using the Super Script III First Strand System (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was performed with Power SYER Green master mix (Applied Biosystems, Foster City, CA). ABI PRISM 7000 sequence detection systems (Applied Biosystems) were used for the measurement. Primer sequences were: *hSPL* sense 5′-TGGAGGTGGATGTGCGGGCAA-3′, antisense 5′-CCCAGACAAGCGTCGACATGAAG-3′; and *hGAPDH* sense 5′-CAGGAGCGAGATCCCTCCAA-3′, antisense 5′-CCCCCTGCAAATGAGCCC-3′. The PCR conditions for *hSPL* and *hGAPDH* were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 62 °C for 1 min and 63 °C for 45 s according to Sobue et al. [24]. In some experiments, semi-quantitative RT-PCR was performed using the same primer set described above [25].

2.3. Western blotting

Western blotting of SPL, Sp1, GATA4 and GATA5 was performed using the anti-hSPL, anti-Sp1, anti-GATA4 and anti-GATA5 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), respectively. Anti-GST antibody was purchased from NAKARAI TESQUE Inc. (Kyoto, Japan). ECL plus western blotting system (Amersham Pharmacia Biotechnology, Buckinghamshire, UK) and Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA) were used for signal detection.

2.4. Cell viability

H1155 and H1299 cells were cultured in triplicate with or without 0.5 mM of DA or 5 μM of ETP. The viable cell number was counted using trypan blue dye exclusion test.

2.5. Cellular ceramide and S1P measurement

The cellular ceramide amount was determined by combined liquid chromatography–tandem mass spectrometry (LC–MS/MS) as described before [26]. Cellular S1P was measured by a metabolic labeling method. Briefly, H1299 and H1155 cells were plated in triplicate 24 h before ^{14}C-serine labeling at the confluence of 70–80%. One day after plating, L-[3-^{14}C]-serine (1.85 MBq/ml) was simultaneously added to the culture medium with or without anti-cancer drugs (0.5 mM of DA or 5 mM of ETP), and further incubated for 1 day. Collected cells were suspended in a solution of 10 ml of 1 N HCl, 400 ml of $CHCl_3$/MeOH/conc HCl (100:200:1, v/v/v), and then 120 ml of $CHCl_3$ and 120 ml of 1 M KCl were added. After centrifugation, the lipids recovered in a lower phase were applied on silica gel thin-layer plate (Merck & Co., Whitehouse Station, NJ, USA), and developed with an authentic S1P in 1-butanol/acetic acid/H_2O (94:1:5, v/v/v). The quantification of ^{14}C-labeled S1P was performed with BAS 2500 systems (Fuji Film Co., Tokyo, Japan). The relative cellular S1P level was calculated based on respective protein level. Data on H1155 cells without anti-cancer drug treatment were regarded as 1.0.

2.6. SPL enzyme activity

Cells were homogenized in the extraction buffer (5 mM 4-morpholinepropanesulfonic acid (MOPS), pH 7.5, 0.25 M sucrose, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), and protease inhibitor cocktail (Sigma)). After centrifugation at 1000 ×g for 10 min, supernatants were transferred to new tubes and protein was determined by the Bradford method using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Berkeley, CA, USA). Standard radiometric SPL assays were performed using [4,5-^{3}H] DHS1P as a substrate, essentially as earlier described [27], but with minor