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Transcriptional regulation of neutral sphingomyelinase 2 gene expression of a human breast cancer cell line, MCF-7, induced by the anti-cancer drug, daunorubicin

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Mg^{2+}-dependent neutral SMases (NSMases) have emerged as prime candidates for stress-induced ceramide production. Among isoforms identified, previous reports have suggested the importance of NSMase2. However, its activation mechanism has not been precisely reported. Here, we analyzed the mechanism of *NSMase2* gene expression by the anti-cancer drug, daunorubicin (DA). DA increased cellular ceramides (C16, C18 and C24) and NSMase activity of a human breast cancer cell line, MCF-7. DA remarkably increased the *NSMase2* message and protein, whereas little change in *NSMase1* and *NSMase3* mRNAs and only a mild increase in *acid SMase* mRNA were observed. Overexpression and a knock down of *NSMase2* indicated that NSMase2 played a role in DA-induced cell death. NSMase2 promoter analysis revealed that three Sp1 motifs located between −148 and −42 bp upstream of the first exon were important in basic as well as in DA-induced promoter activity. Consistently, luciferase vectors containing three consensus Sp1-motifs but not its mutated form showed DA-induced transcriptional activation. DA-treated MCF-7 showed increased Sp3 protein. In SL2 cells lacking Sp family proteins, both Sp1 and Sp3 overexpression increased *NSMase* promoter activity. Increased binding of Sp family proteins by DA to three Sp1 motifs was shown by electrophoresis mobility shift and ChIP assays.

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1. Introduction

Spiegel et al. [1] proposed the sphingolipid rheostat model, where cellular sphingosine 1-phosphate/ceramide ratio determines a cell's fate in areas such as cell growth, cell death, differentiation and cell movement. Sphingomyelinase (SMase) hydrolyzes the membrane sphingomyelin (SM) to ceramide, which is considered to serve as the second messenger. Cellular ceramide then undergoes further metabolism. It could be metabolized to sphingosine, sphingosine 1-phosphate, or other glycosyl sphingolipids. Therefore, ceramide is found to be located at the central part of sphingolipid metabolism [2], and has also been recognized as an important player in the induction of apoptosis [3].

Though the kinetics of SM hydrolysis varies depending on stimuli and cell types, two types of response can be discerned. In the vast majority of cases, stress-induced SM hydrolysis occurs rapidly within minutes [4]. On the other hand, some cytotoxic agents induce a late hydrolysis of SM and ceramide accumulation over a period of several hours or days, suggesting that the SM–ceramide pathway fulfills some function in the execution phase of apoptosis.

SMases have been classified by their optimal pH range. Currently, five types of SMases have been identified; an ubiquitous lysosomal acid SMase, a zinc-dependent secreted acid SMase, a neutral Mg^{2+}-dependent SMase, a neutral Mg^{2+}-independent SMase and an alkaline SMase [5]. Of these, the lysosomal acid SMase and the Mg^{2+}-dependent neutral SMase have emerged as the prime candidates for stress-induced ceramide responses.

Three *NSMases* have been cloned. Among the *NSMases*, *NSMase1* overexpression showed no changes in SM metabolism, whereas it was shown to act as a lyso-PAF phospholipase C in vivo rather than as an

Abbreviations: SMase, sphingomyelinase; SM, sphingomyelin; EMSA, electrophoresis mobility shift assay; ChIP, chromatin immunoprecipitation; DA, daunorubicin; Sp, specificity protein

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NSMase. In fact, it favored lyso-PAF acting as a preferred substrate in vitro [6]. In contrast, overexpression of *NSMase2* was shown to cause a change in SM metabolism, especially an increase in very long chain ceramides, concurrent with a decrease in SM [7]. Furthermore, it has been reported that cellular confluence induced an up-regulation of NSMase activity and *NSMase2* mRNA [8]. *NSMase2* knockout mice showed no deficiency in lipid storage but did exhibit as much as 50% less growth than the wild type, which remained throughout their development [9]. We reported the decreased *NSMase2* message in the bone marrow of myelodysplastic syndromes and acute leukemia compared with normal control bone marrow [10]. Moreover, mutations in *NSMase2* were reported in some leukemia patients [11], suggesting that these changes in the *NSMase2* message are related to leukemogenesis and/or chemosensitivity. *NSMase3*, which shares very little homology with *NSMase2*, has been cloned very recently [12], but its pathophysiological role remains to be determined.

In the current study, we focused on the regulatory mechanism of *NSMase2* gene expression by DA treatment. We previously reported the regulatory mechanism of *ASMase* gene expression induced by all-trans retinoic acid (ATRA) [13]. It is thus of considerable interest to compare the gene expression mechanism between *ASMase* and *NSMase2*, both of which are regarded as two major SMases in the apoptosis process. Our analysis clearly suggested that *NSMase2* is more sensitive to the transcriptional regulation by an anti-cancer drug of DA than other *SMases*. We have, for the first time, provided basic information about the regulatory mechanism of *NSMase2* gene expression, which might prove important for furthering our understanding of the apoptotic process induced by anti-cancer drug treatment.

2. Material and methods

2.1. Cell lines and reagents

A human breast cancer cell line, MCF-7, was cultured in Dulbecco's Modified Eagle's medium (Sigma, St. Louis, MO, USA) with 5% fetal calf serum (JRH Biosciences Inc. Lenexa, KS, USA) at 37 °C in 5% CO_2. A drosophila cell line, SL2, which lacks Sp series transcription factors, was a generous gift from Prof. T. Noguchi (Osaka Otani University, Osaka, Japan). SL2 cells were cultured in Schneider's medium (Invitrogen, Carlsbad, CA, USA) with 10% FCS. Daunorubicin (DA) was purchased from Sigma; Mithramycin A from Fulka (Buchs, Switzerland); pGL3 and pGL4 basic vectors for luciferase assay were from Promega (Madison, WI, USA). siRNA of human *NSMase2* (*hNSMase2*) was purchased from Sigma Genosys (Hokkaido, Japan). The sequence of siRNA of *hNSMase2* was according to Marchesini et al. [8]. siRNA of human *Sp1* and *Sp3* were prepared with the sequence of 5′-GGAUGGUUCUGGUCAAAUATT-3′, 5′-GUUGGGGGAGGUGGAGC-CUTT-3′, respectively [14]. Non-targeting siRNA #1 (Dharmacon) was used as the control scramble siRNA. Sp series expression vectors for SL2 cells, pPac, pPac-Sp1 and pPac-USp3 [15] were originally derived from Prof. G. Suske (Philipps-Universitaet Marburg, Marburg, Germany). For correction of the transfection efficiency of SL2 cells, pPac-RL was used as previously described [16]. Sp1 TransLucent reporter vector (Sp1(2)), which is designed to monitor transcription factor-binding activity of the Sp family through the use of a standard luciferase assay, was purchased from Panomics (Redwood City, CA, USA). Flag-tagged mouse *NSMase2* expression vector was derived from Dr. YA. Hannun (University of South Carolina, SC, USA).

2.2. Western blotting

Western blotting was performed using anti-human NSMase2 antibody (×500 dilution, H-195, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti β-actin antibody (×2000 dilution, Cytoskeleton Inc., Denver, CO, USA) and anti-FLAG antibody (×1000 dilution, Sigma), respectively. Anti-Sp1 (×1000 dilution, PEP2, Santa Cruz) and anti-Sp3 antibody (×1000 dilution, D-20, Santa Cruz) were also used. ECL plus Western blotting system (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) was used throughout the experiments.

2.3. Measurement of viable cells and the analysis of apoptosis

MCF-7 cells were plated in a tissue culture plate of 35 mm in diameter (3.0×10^5/ml) in triplicate 24 h before 0.5 μM DA or other reagents were added. For the detection of apoptotic cells, Annexin-V-FLUOS test (Roche Diagnostics, Indianapolis, IN, USA) was used to detect apoptotic cells (surface phosphatidyl serine (+)) according to the manufacturer's manual. Propidium iodide (final concentration: 1 μg/ml) was also used simultaneously to detect the late apoptotic cells by labeling the nuclei. Stained cells were observed under fluorescent microscopy. Annexin-V positive cells were counted as apoptotic cells. At least 300 cells were counted and the percentage of positive staining was calculated. Viable cell numbers were counted using the trypan blue dye exclusion test. Viable cell numbers on day 0 were regarded as 100%.

Caspase 3 enzyme activity was measured using caspase 3 colorimetric protease assay kit (Medical and Biological Laboratories CO., LTD, (MBL), Nagoya, Japan). The assay is based on spectrophotometric detection of the cleaved product of DEVD-p-nitroanilide, and was measured as the light emission of 400 nm using the microplate reader according to the manual of the manufacturer. The data of control MCF-7 cells were determined as 1.0.

2.4. Real-time and semi-quantitative RT-PCR

Total RNA was extracted using the RNeasy mini kit (QIAGEN, Germantown, MD, USA). The first strand cDNA was prepared with 5 μg of RNA using the Super Script III First Strand System (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was performed with the Power SYBR (Applied Biosystems, Foster City, CA, USA) in duplicate according to Sobue et al. [10]. ABI PRISM 7000 sequence detection systems (Applied Biosystems) were used for the measurements. The primer sequences were shown in Table 1. In addition to GAPDH, β-actin primer set was also used as another internal control. The PCR condition of NSMase2 and GAPDH was 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 63 °C for 45 s. Those of *ASMase*, *NSMase1* and *NSMase3* were the same except for the extension temperature (64 °C for *ASMase*, 62 °C for *NSMase1* and 60 °C for *NSMase3* and β-actin). Semi-quantitative RT-PCR was performed with LA *Taq* (Takara Bio Co, Tokyo, Japan) for NSMase2 and KOD FX *Taq* (Toyobo Biochemical Co, Osaka, Japan) for GAPDH, respectively. Primer sets and annealing temperature used were shown in Table 1 and the PCR cycles were illustrated in Fig. 5c.

2.5. Acid and neutral SMase enzyme activities

Cells were sonicated in 50 mM HEPES—NaOH buffer (pH 7.5) containing a protease inhibitor mixture (Complete-mini, EDTA-free) (Roche Molecular Biochemicals) and 0.5 mM DTT. The assay mixture for the determination of NSMase activity contained 0.1 M Tris/HCl (pH 7.5), 0.1 mM DTT, 10 mM $MgCl_2$, 0.05% Triton X-100, 1.2 M KCl, 20 μM phosphatidylserine, 20 μM [N-methyl-^{14}C] sphingomyelin (20,000 cpm, adjusted by cold sphingomyelin) and 5–10 μg protein of cell homogenate for a total of 50 μl. After incubation for 30 min at 37 °C, the radioactivity of released phosphocholine was determined by a liquid scintillation counter. The assay of ASMase has been described previously [13]. The assay mixture for ASMase contained 0.1 mM acetate buffer, pH 5.8, 1 mM EDTA, 22 μM [N-methyl-^{14}C]

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