Alteration of Gene Expression Induced by *Silurus asotus* Lectin in Burkitt’s Lymphoma Cells

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Silurus asotus lectin (SAL) is a member of the rhamnose-binding lectin (RBL) family, and recognizes globotriaosylceramide (Gb3) on the cell surface of Burkitt’s lymphoma cell lines, such as Raji and Daudi cells. The variation of gene expression in the treatment of both cells with SAL was analyzed using the differential display (DD) method with combination of 16 kinds of arbitrary primers and 3 kinds of anchor primers. Treatment of Raji cells with SAL down-regulated mitochondria-associated granulocyte-macrophage colony-stimulating factor (GM-CSF) signaling molecule (Magmas) gene, and up-regulated N-myc downstream regulated gene (NDRG) 3. On the other hand, treatment of Daudi cells with SAL down-regulated Rad50gene. Since Magmas gene expression was repressed in SAL-treated Raji cells, but did not change in SAL-treated d-threo-1-phenyl-2-decanoylamino-3-morphorino-1-propanol (PDMP)-pretreated Raji cells, it was clear that the expression of Magmas, NDRG3 or Rad50 was regulated by SAL binding to Gb3.

Key words rhamnose-binding lectin; *Silurus asotus*; Burkitt’s lymphoma cell; globotriaosylceramide; differential display method

Rhamnose-binding lectins (RBLs) are widely found in fish eggs.1,2) RBLs preferentially agglutinate rabbit and human type B erythrocytes, and the hemagglutinating activity is strongly inhibited by l-rhamnose and α-galactoside rather than β-galactoside. Additionally, they do not require divalent cations such as Ca2+ and Mg2+ for their hemagglutinating activity.1,3,4) RBL isolated from catfish (*Silurus asotus*) eggs, SAL, is a 32-kDa protein and consists of three tandem repeat domains of 95 amino acid residues.5) SAL has a potent affinity to Gal α-linked carbohydrate chains of not only glycoproteins but also glycosphingolipids such as globotriaosylceramide (Gb3) from the results of surface plasmon resonance spectrometric analysis (Hosono M., Sugawara S., Takayanagi M., Nitta K., unpublished observation).

Since it is well-known that the B subunit of Shiga toxin by itself participates in signal transduction and induces apoptosis against Gb3-expressing (Gb3⁺) Burkitt’s lymphoma cells, such as Raji and Daudi cells,5) we examined whether or not the binding of SAL to Gb3 induces apoptotic cascade. In the course of this study, we found that: 1) SAL bound to Gb3 on the surface of Raji cells, 2) SAL had the ability to induce externalization of phosphatidylserine through activation of multidrug resistance 1 P-glycoprotein (MDR1 P-gp) and then incorporation of propidium iodide, and 3) SAL induced cell shrinkage but not cell death.7) That is, it was concluded that the binding of SAL to Gb3 leads the cells to an early stage of apoptosis, but does not commit Raji cells to late apoptotic status.7) However, it is still unknown what kind of molecules participate in signal transduction on the binding of SAL to Gb3-expressing Raji cells.

In this study, we used Gb3-expressing Raji and Daudi cells to investigate whether: 1) the variation of gene expression in the treatment of both cells with SAL can be analyzed using the differential display (DD) method; 2) treatment of Raji cells with SAL down-regulates mitochondria-associated granulocyte-macrophage colony-stimulating factor (GM-CSF) signaling molecule (Magmas) gene, and up-regulates N-myc downstream regulated gene (NDRG) 3; and 3) treatment of Daudi cells with SAL down-regulates Rad50 gene. Furthermore, it has become apparent that the binding of SAL to Gb3 regulates the expression of Magmas, NDRG3 or Rad50.

MATERIALS AND METHODS

Materials SAL was isolated by sequential chromatographies on DE23 (Whatman) anion exchange and d-galactose-Sepharose 6B columns as described previously.5)

Cell Lines Burkitt’s lymphoma Raji and Daudi cell lines were obtained from the Cell Resource Center of the Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). Human erythroleukemia K562 cell line was obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). These cell lines were cultured in RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10 % fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml), and maintained at 37 °C in 95% air and 5% CO2 atmosphere.

Raji and Daudi cells are Gb3-expressing cells, whereas K562 cells are Gb3-negative cells.5) Gb3-deficient Raji and Daudi cells were prepared by the method described below: Raji and Daudi cells (1×10⁵) were cultured on 24 well-plates and treated with a glycolipid biosynthesis inhibitor, d-threo-1-phenyl-2-decanoylamino-3-morphorino-1-propanol (PDMP, final concentration: 20 μM for Raji, 10 μM for Daudi) (Matreya, Pleasant Gap, PA, U.S.A.) for 4 d and 6.5 d, respectively.9—11) The percentage of Gb3 positive cells was determined by flow cytometry. The expression levels of Gb3 on the surface of Raji, K562 and Daudi cells were 81.5, 72.0 and 0.9%, respectively. On the other hand, the levels of Gb3 on PDMP-treated Raji and Daudi cells were 21.3 and 26.0%, respectively (Kawano T., Sugawara S., Hosono M., Takayanagi M., Taka H., Fujimura T., Murayama K., Hakomori S., Nitta K., unpublished observation).

Differential Display Method mRNA differential display (DD) technology was developed by Pardee and Liang.12) Raji...
and Daudi cells (1×10⁶/ml) were cultured in the presence of SAL (100 μg/ml) at 37 °C for 24 h in a CO₂ incubator. Total RNA was prepared using Trizol (Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions. The reverse transcriptase (RT) reaction was performed using a RNAspectra™ Green kit (GenHunter Corporation, Nashville, TN, U.S.A.) with total RNA (0.2 μg) and anchor primers (see Table 1). The polymerase chain reaction (PCR) was then performed using RNAspectra™ Green kit with arbitrary primers (2 μM) and anchor primers (2 μM) (see Table 1), and RT reaction product (cDNA) as a template. The PCR amplification was performed using the Progene thermal cycler apparatus (Techne Inc., Princeton, NJ, U.S.A.) in a total volume of 20 μl with 40 amplification cycles each at 94 °C for 30 s, 40 °C for 120 s, and 72 °C for 60 s. The final elongation step was carried out at 72 °C for 7 min. The resulting products were subjected to polyacrylamide gel electrophoresis (PAGE) and analyzed by FIA-3000G fluorescence imaging analyzer (Fujifilm, Tokyo, Japan). Each reaction was performed using RNAs obtained from two independent experiments. Bands unchanged consistently over three experiments were excised from the gel and cloned into the pGEM-T easy vector (Promega, Madison, WI, U.S.A.). The fragment sequence of each band was determined using a Dye terminator cycle sequencing kit, CEQ8000 capillary sequencer (Beckman Coulter, Fullerton, CA, U.S.A.) and Applied Biosystems 3130 genetic analyzer (Applied Biosystems, Foster City, CA, U.S.A.).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction Total RNA was extracted from SAL-treated and -untreated Raji, Daudi and K562 cells using Trizol (Invitrogen) as described above. The RT reaction was performed using ReverTra Ace (Toyobo, Osaka, Japan) with total RNA (1 μg) and oligo-dT₂₅ primers. The first-strand cDNA (1 μl) was used as a template for the quantitative RT-PCR (qRT-PCR). qRT-PCR was performed using a Thermal Cycler Dice™ real time system (Takara Bio Inc., Ohtsu, Japan) or a LightCycler® 480 real-time PCR system (Roche Diagnostic GmbH, Mannheim, Germany). The specificity of PCR was confirmed by the molecular weight of PCR product and melting curve analysis at each point of the reaction. Relative quantities of interesting genes were standardized against the quantity of RNA coding β-actin in each sample. Gene specific primers were as follows: Magmas, 5’-GCACAGCAGATTCTCAACGTGTC-3’, 5’-GAGCCACCCACGGAATTTATCA-3’; NDRG3, 5’-GGACCATGATTGATTGTGTGACCCAG-3’, 5’-GGTTCATGGGGTCTCCAGCA-3’; Rad50, 5’-CAGGTGACACCACCACCTTTG-3’, 5’-CTCCTGTGAGTGAGGCTTGGCT-3’; β-actin, 5’-TGCCACCAGCAATGAGAA-3’, 5’-CTAAGTCATAGTCCGCTAGAAGCA-3’. Each experiment is representative of at least two independent experiments.

Computational Analysis Database analysis for the genes of Magmas, NDRG3 and Rad50 was performed using the advanced BLAST search.13)

Statistical Analysis Results are reported as the mean of percentage±standard error (S.E.) of the mean. Two-tailed Student's t-tests or ANOVA were used as appropriate for statistical analysis. Only p values <0.05 were considered to be statistically significant.

| Table 1. Sequences of Anchor Primers and Arbitrary Primers |
|-----------------|-----------------|
| Anchor primer   | H-T_1G          |
|                 | H-T_1A          |
|                 | H-T_1C          |
| Arbitrary primer| H-AP 1          |
|                 | H-AP 2          |
|                 | H-AP 3          |
|                 | H-AP 4          |
|                 | H-AP 5          |
|                 | H-AP 6          |
|                 | H-AP 7          |
|                 | H-AP 8          |
|                 | H-AP 9          |
|                 | H-AP 10         |

RESULTS

Alteration of Gene Expressions in SAL-Treated Burkitt's Lymphoma Raji and Daudi Cells and Identification of Their Genes SAL bound selectively to Gb3 on the cell surfaces of Raji and Daudi cells, and agglutinated them.7) However, the cytochemical actions of SAL against Gb3-expressing cells are still unknown. We thus examined whether the expression fluctuation of molecules was dependent on binding of SAL to Gb3-expressing cells or not. RNA isolated from Raji and Daudi cells treated with SAL for 24 h was analyzed using the DD method. The forty-eight kinds of PCR were performed using all combinations of 16 kinds of arbitrary primers and 3 kinds of anchor primers (see Table 1) in each cell line. After amplification by PCR, the products were subjected to PAGE. Significant differences were detected in 43 kinds of bands using the DD method (data not shown).

To confirm whether the detection of candidate genes was reproducible or not, we performed supplementary RT-PCR and DD. As shown in Fig. 1, we confirmed that the alteration of expression was reproducible in three bands in SAL-treated Burkitt's lymphoma cell lines. After these bands were cut out from the gel and cloned, we analyzed the DNA sequence of the bands. DNA fragment sequences of each band are shown in Fig. 2. From the results of the homology search,13) DNA sequences of Bands A, B and C corresponded to a fragment (258—527) of Magmas gene (Accession No: NM_032013) and a fragment (917—1207) of NDRG3 gene (Accession No: NM_016069), and a fragment (2317—2552) of Rad50 gene (Accession No: U63139), respectively.

We then performed qRT-PCR to investigate whether these gene expressions were regulated by treatment with SAL (Fig. 3). The qRT-PCR analysis revealed that the mRNA level of Magmas decreased to about one-sixth and that of NDRG3 increased by 2.5 times, whereas no expression level of Rad50 changed in SAL-treated Raji cells (Figs. 3A—C), and that the level of Rad50 decreased to about one-half, whereas the expression levels of Magmas and NDRG3 decreased with no significant in SAL-treated Daudi cells (Figs. 3D—F). In the
Fig. 1. Detection of Candidate Gene by Differential Display Analysis

Cells were treated with or without SAL. DD analysis was performed using RNAspectra Green kit as described in Materials and Methods. The cDNA sub-populations amplified using the short arbitrary primers in combination with the anchored oligo-dT primers are distributed on DNA sequencing gels. For Raji cells: total RNAs from SAL-treated (lanes; 2, 4, 6, 8) and -untreated (lanes; 1, 3, 5, 7) Raji cells were submitted to PCR using H-AP5 and H-T11A primers (lanes; 1, 2), H-AP4 and H-T11G primers (lanes; 3, 4), H-AP7 and H-T11A primers (lanes; 5, 6), and H-AP8 and H-T11A primers (lanes; 7, 8). Arrows and asterisks are desired bands. Indications of plus (+) and minus (−) are the cDNA sequences indicated by solid line and dotted line, respectively. This figure shows typical data in two experiments.

Fig. 2. cDNA Sequences of Candidate Genes

Nucleotide sequences (a, b, c) were derived from Bands A, B and C of Fig. 1, respectively. The nucleotide sequences indicated by solid line and dotted line are the arbitrary primer and anchor primer, respectively (see Table 1).

case of K562 cells, no expression level of Magmas, NDRG3 and Rad50 was changed without regard to treatment with SAL (Figs. 3A—I).

Regulation of Gene Expressions of Magmas, NDRG3 and Rad50 by the Binding of SAL to Gb3 To confirm whether the binding of SAL to Gb3 caused down-regulation of Magmas and Rad50 expression and up-regulation of NDRG3 expression, we examined the effect of treatment with SAL on the expression of these genes in PDMP-Raji, and PDMP-Daudi cells using qRT-PCR with specific primers. Although the down-regulation of Magmas gene and up-regulation of NDRG3 gene were observed in SAL-treated Raji cells (Figs. 3A, B), the slight differences without significant expressions of these genes were observed in SAL-treated PDMP-Raji cells (Fig. 4). Furthermore, the down-regulation of Rad50 gene was observed in SAL-treated Daudi cells (Fig. 3F), while no expression change was observed in SAL-treated PDMP-Daudi cells (Fig. 4). These results suggest that the mRNA expressions of Magmas, NDRG3 and Rad50 were regulated by SAL binding to Gb3.

DISCUSSION

In this study, we attempted to elucidate the variation of gene expression caused by the treatment of Gb3-expressing Burkitt's lymphoma cell lines with SAL, and to analyze it using the DD method. Figures 1, 2 and 3 show that the down-regulated and up-regulated genes were identified as Magmas and NDRG3, respectively, in SAL-treated Raji cells. On the other hand, in SAL-treated Daudi cells, Rad50 gene was down-regulated.

Jubinsky et al. reported that: 1) Magams gene was rapidly induced at the conversion from IL-3 to GM-CSF in the composition of culture medium; 2) Magmas protein localized to mitochondria; and 3) the growth of PGMD1 cells with reduced Magmas expression appeared normal in IL-3 but not in GM-CSF. GM-CSF receptor is composed of α and β subunits. Each of the subunits, α and β, is responsible for the specificity of ligand binding and for the signal transduction, respectively. We confirmed that the β subunit but not the α subunit was expressed in Raji cells (Kawano T., Sugawara S., Hosono M., Tatsuta T., Nitta K., unpublished observation). Consequently, GM-CSF cannot bind to the surface of Raji cells. Magmas was derived from Raji cells (R-Magmas) was constitutively highly expressed in GM-CSF-unstimulating Raji cells (see Fig. 3). Results of qRT-PCR data showed that expression of the R-Magmas gene was observed in SAL-unstimulated cells, but treatment with SAL caused a sharp decrease (Fig. 3). On the other hand, the difference of Magmas expression in PDMP-treated Raji cells was barely observed in the treatment with or without SAL (Fig. 4). That is, the binding of SAL to Gb3 resulted in the reduction of Magmas expression.

Magmas could be involved in the increase of anaerobic metabolism, resistance to apoptosis or altered growth factor sensitivity in prostate cancer tissues. Magmas is essential for Caenorhabditis elegans development, and the homozygous deletion of Drosophila homolog causes death at the first larval instar stage. Similarly, the disruption of MIA1, a yeast homolog, is lethal in Saccharomyces cerevisiae. Additional yeast studies showed Magmas to be involved in the transport of proteins in mitochondria; and 3) the growth of PGMD1 cells with reduced Magmas expression appeared normal in IL-3 but not in GM-CSF. Furthermore, it is known that GM-CSF-dependent proliferation is significantly impaired in PGMD1 cells with low levels of Magmas protein using anti-sense cDNA constructs of Magmas. These results suggest that Magmas could correlate growth factor signaling pathways with mitochondrial functions, that is, the relationship between apoptosis and respiratory activity.

Because SAL binds to Gb3-expressing Raji cells and leads the cells to an early stage of apoptosis, Magmas may be involved in SAL-induced early stage apoptosis. Not only Gb3...
but also multiple transducer molecules (e.g. c-Src, Rho, Ras, FAK, Lyn etc.) were localized in glycosphingolipid-enriched microdomain (GEM).\textsuperscript{21,22} GM-CSF receptor was important for proliferation, differentiation and survival.\textsuperscript{23} Furthermore, it appears that the activation of PI3K dependent on GM-CSF is mediated by the interaction between $\beta$ chain and Src-tyrosine kinase Lyn.\textsuperscript{24} In view of these facts, SAL may regulate the growth of Gb3-expressing Raji cells via suppression of tyrosine phosphorylation.

NDRG3 and NDRG1 together comprise the NDRG family.\textsuperscript{15} N-myc/Max complex can repress NDRG1 promoter activity and so does c-myc/Max complex, indicating that NDRG1 expression might be repressed during malignant transformation of cells of which N-myc or c-myc activity is augmented.\textsuperscript{25} In contrast to NDRG1, NDRG2 and 3 were not under negative regulation by N-myc.\textsuperscript{15} Thus, it is possible that NDRG3 was regulated by signal transduction through the binding of SAL to Gb3.

The Rad50 complex consists of two highly conserved proteins, Mre11 and Rad50, and a third protein, either Xrs2 in budding yeast or Nbs1 in mammals.\textsuperscript{26,27} This complex 1) mediates diverse functions in several aspects of DNA metabolism, including DNA double-strand break (DSB) repair and telomere maintenance, 2) is implicated in two different repair processes of DSB: homologous recombination (HR) and

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Fig. 3. Fluctuation of Candidate Gene Expression in SAL-Treated Raji (A, B, C), Daudi (D, E, F) and K562 cells (G, H, I)

Total RNAs were extracted from SAL-treated (+) and -untreated cells (−) cells. qRT-PCR was performed using specific primers for Magmas (A, D, G), NDRG3 (B, E, H), Rad50 (C, F, I) and β-actin (control gene), respectively. The copy numbers of candidate gene mRNA were standardized against that of β-actin in each sample. Data are expressed as relative values on the basis of the value of β-actin. Each value represents the mean value ± S.E. for two or three different experiments performed in quadruplicate. *$p<0.05$ (untreated control versus treatment with SAL).

Fig. 4. Alteration of Gene Expression in SAL-Treated PDMP-Raji and PDMP-Daudi Cells

Total RNAs were extracted from SAL-treated (+) and -untreated PDMP-Raji and PDMP-Daudi cells (−). qRT-PCR was performed using specific primers for Magmas (A), NDRG3 (B) and β-actin (control gene), respectively. Data are analyzed in the same manner as described in the legend of Fig. 3.
nonhomologous end joining (NHEJ), and, in addition, 3) is required for the G2/M phase DNA damage checkpoint after DSB induction. Because Rad50 gene was suppressed by treatment with SAL, we speculated that the repair processes of DSB were regulated by the binding of SAL to Gb3. Consequently, it may be concluded that the expression of Magmas and the other two genes is regulated by SAL binding to Gb3 localized in GEM.

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