別紙３
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分担研究報告書

発現糖鎖解析、糖鎖修飾分子合成および抗糖鎖抗体作製

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研究要旨：分化・がん化により細胞に発現する糖脂質は多様に変化する。これまで細胞に発現する糖脂質の解析には薄層クロマトグラフィー(TLC)やTLC免疫染色などが多く使用されてきたが、化学発色による感度の低さや移動度による判別の不正確さ、抗体のない糖脂質では低発現のものは同定が困難であるなど、不便な点が多くあった。我々はその糖脂質の解析にキャピラリー高速液体クロマトグラフィー（Cap LC)と構造解析が高感度に行うことが可能なマススペクトロメトリー（MS）を組み合わせたLC/MSを使用し細胞に発現糖脂質を網羅的に解析する。
本年度は内在性糖脂質を効率よくLC/MSで解析する方法を確立するために、新たに開発されたジルコニア固相を用いて細胞から得られた脂質画分からLC/MSによる分析に大きな障害となるリン脂質を効率よく除去する方法を確立し、糖脂質を無傷なまま効率よく回収してLC/MSで分析する手法を確立した。

A．研究目的

糖鎖は核酸・蛋白質に続く第３の生命鎖といわれ、細胞に発現する糖鎖は、細胞の成熟やがん化により発現パターンが大きく変化することが知られている。これまでこの糖鎖の解析は薄層クロマトグラフィー（TLC）やレクチンなどを用いて行われてきたが、感度や特異性が充分ではなかったため、詳細な解析は比較的大量に存在するものに限られていた。一方マススペクトロメトリーは微量な生体分子を網羅的に高感度で検出できる手段であり、近年蛋白質を網羅的に解析するプロテオミクスに大いに利用されている。また糖鎖プライマー法は細胞をいわば糖鎖工場と見立てて培養液中に糖鎖を大量に生産させる技術である。

本研究ではこのマススペクトロメトリーと糖鎖プライマー法を網羅的発現糖鎖解析（グライコミクス）に利用して、ヒト未分化細胞（白血病細胞やヒト間葉系幹細胞）に発現している糖鎖を網羅的に解析し、これまで微量しか発現していなかったため同定されていなかった糖鎖を同定し、細胞の糖鎖発現パネルを作製すると共に、その発現糖鎖の中から診断に有用な糖鎖を検索し、将来的に診断・治療応用することを目指す。昨年度はLC/MSを用いて糖脂質糖鎖に加え糖蛋白質N結合型糖鎖を分離・同定・構造解析する系を構築した。本年度は細胞内在性糖脂質をLC/MSで解析する際に夾雑物として大きな問題となるリン脂質を効率よく除去する方法を確立し、患者検体のような微量な細胞でも発現糖脂質をLC/MSで網羅的に解析する方法を確立する。

B．研究方法

１．ジルコニア固相によるリン脂質の除去

ジルコニア固相とはシリカゲル担体にジルコニアを結合させた物である。ジルコニアの4d電子軌道にはリン酸基が効率よく結合することが報告され、リン酸化蛋白質、リン酸化ペプチドなどを回収するのに利用され、リン酸化された蛋白質を解析するプロテオミクスなどに利用されていた。また、血清プロテオミクスにおいては、血中に微量に存在するリン脂質がターゲット蛋白質由来のペプチドのイオン化を抑制（イオンサプレッション）することが知られ、それを可能な限り取り除くために利用されている。そこで本研究においても細胞に含まれるリン脂質のみをジルコニア固相に吸着除去させ、糖脂質を有効に回収することを試みた。

ジルコニアには糖鎖に含まれるシアル酸とも結合する可能性があり、リン脂質と共に除去されてしまう危険性がある。そこでジルコニアに対してリン酸基と同様にルイス酸・塩基として働くギ酸、クエン酸を様々な濃度で糖脂質と共にメタノール中に共存させ、リン酸基特異的に結合・吸着させる条件を検討した。

2．ジルコニア固相による糖脂質のLC/MSによるイオンサプレッション防止効果の確認

細胞に発現するリン脂質で最も多い物はホスファチジルコリン（PC）である。TLCや順相HPLCでPCと比較的近い挙動を示す糖脂質として、GM3とGb4Cerがある。ACHN細胞から常法に従って抽出した総脂質画分試料、アルカリ分解によってリン脂質を分解した試料、ジルコニア固相によってリン脂質を除去した試料をそれぞれLC/MSで分析し、GM3とGb4Cerに相当するm/z値でクロマトグラムを描き、ピーク面積を比較した。

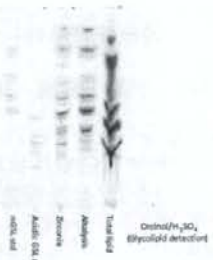
3．O-アセチルシアル酸の確認

これまで細胞に発現する糖脂質をTLCなどで分析する場合、大量に存在するリン脂質を除去するため、アルカリ分解という手法が用いられてきた。昨年度の糖鎖プライマー法を用いた実験から、神経芽腫細胞には糖脂質にO-アセチルシアル酸を発現しているものが明らかになっている。糖鎖プライマー法では、アルカリ分解を行わず、培地より糖鎖を回収できるという利点があったため、そのような発見につながった。本年度は、神経芽腫細胞CHP134より常法に従い脂質を抽出し、ジルコニア固相でリン脂質を除去した試料について内在性糖脂質においてもO-アセチルシアル酸が無傷で残り、発現を確認することができるか検証した。

C．研究結果

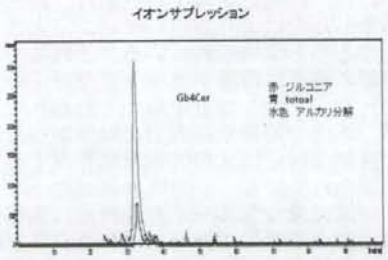
1．ジルコニア固相によるリン脂質の除去

ACHN細胞より抽出した総脂質試料、アルカリ分解によってリン脂質を分解した試料、ジルコニア固相によってリン脂質を除去した試料をそれぞれHPTLCにアプライして分析した。ルイス酸・塩基としてはクエン酸0.1%を共存させた。結果、総脂質ではTLC上におけるバンドが夾雑物（リン脂質）により大きく乱れたのに対し、ジルコニア固相でリン脂質除去した試料、これまでのようにアルカリ分解でリン脂質を分解させた試料はバンドが乱れず、ジルコニア固相によるリン脂質除去は非常に有効であることが明らかとなった。



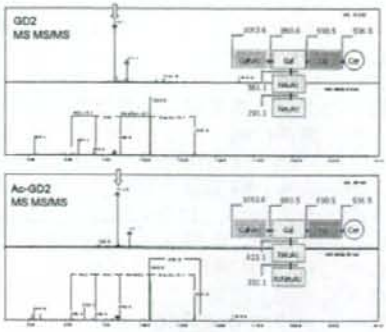
2．ジルコニア固相による糖脂質のLC/MSによるイオンサプレッション防止効果の確認

ACHN細胞から得られたGb4CerとGM3に関してExtracted Ion Chromatogramを各試料に対して描かせたところ、ジルコニア固相でリン脂質除去した試料がもっとも大きなピーク面積が得られた。これはLC/MSにおいてGb4CerとGM3とほぼ同じ時間にLCで溶出するPCの夾雑がジルコニア固相により抑えられたためと考えられる。



3．O-アセチルシアル酸の確認

CHP134細胞より得られた脂質画分からジルコニア固相によりリン脂質を除去しLC/MSにより分析した。Oアセチルシアル酸を持つGD2のm/z値のイオンに対し、MS/MSスペクトルを取ったところ、Oアセチルシアル酸に相当するフラグメントイオンを確認することができたことから、ジルコニア固相ではOアセチルシアル酸を含んだガングリオシドを無傷なまま回収することができることが明らかとなった。



D．考察

これまで糖脂質を含む糖鎖の分析にはTLCやレクチンなどをプローブとした分析法が多用されてきたが、感度に大きな問題点があった。中でも糖脂質分析に用いられるTLCでは、細胞から有機溶媒で抽出した総脂質を直接載せてして分離し、糖のみを検出する試薬を噴霧して検出する方法が採られてきたが、この方法ではリン脂質のTLC上での挙動が糖脂質と非常に近いため、しばしば結果に影響を与えることがあることに加え、感度的にも問題があった。これまで糖鎖プライマー法によって得た糖鎖をLC/MSを使用することにより網羅的に解析する方法を確立したが、内在性糖脂質についても実際に存在しているか確認する必要があった。しかし従来の方法では内在性糖脂質を網羅的に解析する方法はなく、酸性糖脂質をイオン交換クロマトグラフィーで分画するなどの煩雑な方法が必要だったため、LC/MSによる分析については中性糖脂質を含んだ状態で定量的に糖脂質を回収することは困難であった。ジルコニア固相を用いた方法を採ることによってLC/MSによる各試料間の内在性発現糖脂質のクラスタリング解析がより正確に行えるようになった。

E．結論

新たに開発されたジルコニア固相を用いることで、細胞から回収される脂質画分よりLC/MSによる分析で非常に問題となる夾雑物であるリン脂質を有効に除去することが可能となり、臨床検体のような微量な細胞からでも細胞に発現する糖脂質の網羅的な解析が可能となり、発現糖脂質によるクラスタリング解析が可能になった。

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G．知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得
該当なし

2. 実用新案登録
該当なし

3.その他
該当なし

III　研究成果に関する一覧表

研究成果の刊行に関する一覧表

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著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
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IV　研究成果の刊行物・別冊

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SHORT COMMUNICATION

The detergent-insoluble microdomains, rafts, can be used as an effective immunogen

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Abstract Detergent-insoluble microdomains, or rafts, act as a platform to transduce signals from the extracellular space into the cytoplasm. In the process of developing monoclonal antibodies against raft molecules for the purpose of studying the molecular mechanism of raft-mediated signaling, we observed the uniqueness and certain advantages of immunization with rafts. Simple subcutaneous injection of mice with a phosphate-buffered saline (PBS) suspension of rafts without mixing with Freund's adjuvant made it possible to increase the titer of antiserum reacting with raft components. Interestingly, injection of rafts prepared from certain specific cell lines induced monoglycolipid-specific antibodies. Furthermore, antibodies were produced by raft-immunization of even syngeneic mice. Our findings suggest that this phenomenon does not represent a breakdown of immunological self-tolerance, but typical immune reactions accompanying the class switch from IgM antibodies to IgG antibodies.

Keywords Raft · Antibody · Immune Response · Monoglycolipid-specific · Syngeneic antigen

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Introduction

There is evidence that detergent-insoluble microdomains, or rafts, are important in signal transduction, because a variety of signaling molecules, such as Src-family kinases, heterotrimeric G proteins, and GPI-anchored proteins, are concentrated in rafts. We have previously shown that the binding of Shiga-toxin (Stx) to the globotriaosylceramide (Gb3[1]) in rafts temporally activates the Src-family kinase Yes in human renal cancer cell line ACHN [1]. In order to study the downstream signaling mechanism after Stx binding to Gb3, we attempted to develop monoclonal antibodies against components of rafts prepared from ACHN cells and established several clones [2].

In the process we observed the uniqueness of immunization using raft suspensions. Before immunizing animals in an attempt to induce antibody production, antigen solutions or cell suspensions are generally mixed with Freund's adjuvant to obtain an oil emulsion, whereas we succeeded in raising antibody titer by the raft immunization method without mixing them with adjuvants. Interestingly, two thirds of the clones obtained reacted with lipid components of the raft, and further analysis showed that all of the lipid-reactive clones recognized monosialosylgalactosylgloboside (sialylGb5).

To ascertain whether raft immunization always induces monospecific antibodies that recognize a certain glycolipid, we immunized mice with rafts prepared from several cell lines and examined the glycolipid antigens recognized by the antibodies induced. In this paper we report that injection with

[1] Glycosphingolipids are abbreviated according to the recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature. http://www.chem.qmul.ac.uk/iupac/misc/glylp.html.

rafts prepared from certain specific cell lines can induce the production of monoglycolipid-specific antibodies and that raft immunization can induce antibody production even in syngeneic mice.

Materials and methods

Cell culture and antibodies Human renal cancer cell line ACHN, human T-cell leukemia cell lines Jurkat and MOLT-4, and mouse myeloma cell line P3U1 were purchased from the American Type Culture Collection, and the African green monkey kidney cell line Vero was a gift of Dr. T. Takeda of the Department of Infectious Diseases Research, National Children's Medical Research Center, Tokyo, Japan. Anaplastic large cell lymphoma Karpas 299 cells [3] were gifted by Dr. K. Kikuchi of Sapporo Medical University, School of Medicine, Sapporo, Japan. Human pre-B ALL cell line NALM-6, mouse T lymphoma cell line EL4, mouse melanoma cell line B16F1, and mouse leukemia cell line RL2 were obtained from the Institute of Development, Aging and Cancer of Tohoku University, Sendai, Japan. The ACHN cells, Vero cells, and B16F1 cells were cultured in Dulbecco's modified Eagle's medium (Sigma Chem., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Cansera International Inc., CCT, Canada). All other cell lines were cultured in RPMI 1640 supplemented with 10% FBS. The NZB/WF1 serum was a kind gift of Dr. S. Kon of the Institute of Genetic Medicine, Hokkaido University, Sapporo.

Raft preparation Rafts were prepared as described previously [2]. Briefly, packed cells were homogenized in 1% Triton lysis buffer (1% Triton X-100, 25 mM Tris–HCl buffer, pH 7.5, 0.15 M NaCl) by 20 strokes with a hand-driven Teflon glass homogenizer. Cell lysates, sucrose concentration of which was adjusted to 40% with 85% sucrose solution, were placed on the bottom of an ultracentrifuge tube, and a 5/30% discontinuous sucrose gradient was formed over the sample. After centrifugation at 39,000 rpm for 18 h at 4°C in a Beckman SW 40Ti rotor, rafts were recovered as visible bands at the interface between 5 and 30% sucrose solution. After several washes with PBS, raft suspensions in PBS were stored at −30°C until used.

Immunization of mice Rafts prepared from 1.2×10^6~1.5×10^8 cells or 10^7 cells irradiated at 10 Gy were suspended in 100 μl of PBS. They were subcutaneously injected into mice in triplicate, followed by three booster shots at 1-week intervals. Five days after the final injection, a peripheral blood specimen was collected from the mice, and the level of antibodies against rafts was evaluated.

TLC immunostaining Lipids were prepared from packed cells as previously described [4] and separated on a Silica gel 60-precoated HPTLC aluminium sheet (Merck, Darmstadt, Germany) with a solvent system consisting of chloroform/methanol/water containing 0.2% $CaCl_2$ (5:4:1, *v/v*). After drying, the TLC plates were coated with 0.1% polyisobutylmethacrylate (Sigma-Aldrich, Milwaukee, WI) in cyclohexane and blocked with 1% bovine serum albumin (BSA) in PBS. The plates were probed with anti-sera (diluted to 1:500 in 1% BSA in PBS) for 1 h at room temperature. After three washes with PBS for 5 min each, horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulins G+M (DAKO, A/S, Denmark) at a 1:2,000 dilution ratio were used as the second antibody. The antibodies that bound to the plates were visualized with enhanced chemiluminescence reagent Super Signal (Pierce, Rockford, IL) and detected with a luminescent imaging analyzer, LAS-1000 (Fuji Film, Tokyo, Japan). To compare the amounts of antibodies in the sera, the intensity of chemiluminescence was measured with Image Gauge analysis software equipped to LAS-1000 and shown as Photo Stimulated Luminescence (PSL).

Dot-blot immunostaining assay The ACHN rafts were dot-blotted on a PVDF membrane (Millipore Corp., Bedford, MA) and immunostained as described previously [2] with a slight modification. The dots were probed with antisera (diluted to 1 in 500 with 1% BSA in PBS) for 1 h at room temperature. After four washes with PBS containing 0.025% Tween 20 (PBS-Tween), the membranes were treated with HRP-conjugated rabbit anti-mouse IgG antibodies specific to Fcγ fragment and HRP-conjugated goat anti-mouse IgM antibodies specific to μ chain (Jackson Immuno Research Laboratories, West Grove, PA) to detect IgG and IgM, respectively. The antibodies that bound to the membrane were visualized with enhanced chemiluminescence (ECL Western blotting system; Amersham Pharmacia Biotech. UK Ltd., Buckinghamshire) and detected by a luminescent imaging analyzer as mentioned above.

Flow cytometry Cells were harvested from culture plates, and after incubating with the antisera (diluted to 1:100 in RPMI medium containing 5% FBS and 0.1% NaN_3) for 1 h on ice, they were treated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulins (Jackson Immuno Research Laboratories) at a 1:50 dilution ratio and analyzed by flow cytometry (EPICS-XL, Beckman-Coulter, Fullerton, CA).

Measurement of anti-ss and -dsDNA antibodies in sera by ELISA The ELISA was performed as described by Iizuka *et al.* [5] with a slight modification by using calf thymus ssDNA (Sigma) and salmon sperm dsDNA (Sigma). For

the substrate solution, 120 µl of 0.1 mg/ml 3,3′,5,5′-tetramethylbenzidine (Dojindo, Kumamoto, Japan) solution in *N,N*,dimethylformamide and 1.3 µl of 30% H_2O_2 was mixed with 7.88 ml of 0.1 M sodium acetate buffer, pH 5.5, immediately before use. A 0.5 µg amount of ssDNA or dsDNA dissolved in 50 µl of PBS were allowed to dry in a flat 96-well NUNC-IMMUNO Plate (Nunc, Roskilde, Denmark). Wells were blocked with 3% BSA in PBS and washed with PBS-Tween. A 50 µl of the serum (diluted to 1:100) was added to a well in triplicate, and allowed to stand at room temperature for 2 h. After five washes with PBS-Tween, HRP-conjugated donkey anti-mouse µ chain antibodies or rabbit anti-mouse γ chain antibodies (Jackson Laboratory; diluted to 1:2,000) in 1% BSA in PBS was added to each well and incubated for 1 h at room temperature. After three washes with PBS-Tween, 50 µl of substrate solution was added, and the plates were incubated at room temperature until the solution turned yellow. The reaction was stopped by adding 50 µl of 2 M H_2SO_4, and absorbance at 450 nm was measured with a microplate reader (Model 550 Bio-Rad, Richmond, CA).

Results and discussion

The antisera obtained from Balb/c mice in response to subcutaneous injection of rafts prepared from a variety of cell lines were examined by TLC-immunostaining to analyze the reactivity of the antibodies against glycolipids. Both ACHN cells and Vero cells are derived from kidney and express globoseries glycosphingolipids, whereas Karpas cells predominantly express LacCer, and EL4 cells mainly express GM2 and GD2 (Fig. 1a). The antisera obtained by injection with ACHN rafts and Vero rafts were found to uniquely bound to sialylGb5, suggesting the development of mono-specific antibodies against sialylGb5 (Fig. 1b). As we previously showed, ACHN cells contain comparable amounts of Gb3, Gb4, Gb5, and sialyGb5, suggesting that the sialylGb5 of ACHN cells is strongly immunogenic [2]. The antisera obtained by injection with Karpas rafts were also found to specifically bind to a single glycolipid that has not yet been identified. Since the glycolipid was stained with resorcinol and not bound by choleratoxin even after digestion with *Clostridium perfringens* sialidase (data not shown), it is suggested that this antigen is a sialylated non-ganglioseries glycolipid. The observation that the anti-Karpas rafts antisera did not bind to any glycolipid extracted from mouse brains in which various kinds of gangliosides are abundantly contained (data not shown) should support this idea. The antisera obtained by injection with EL4 rafts were found to uniquely react with GD2. However, when antisera obtained by injection of rafts prepared from the other cell lines, *i.e.*, B16F1, P3U1, RL-2, Molt 4, Jurkat, or NALM-6, were tested, no such monoglycolipid-specific reactivity was observed (data not shown). These findings indicate that immunization with rafts prepared from some specific cell lines can induce the development of monoglycolipid-specific antibodies. Since we obtained identical results in a similar experiment in C57BL/6 mice (Fig. 1c), the development of monoglycolipid-specific antibodies is a common feature of immunization of these cell lines with rafts and not a phenomenon specific to a certain strain of mice.

Immunization of mice with a suspension of whole cells is one of the ways that is often used to obtain monoclonal antibodies against cell surface molecules [6, 7]. We therefore investigated whether whole-cell immunization is capable of inducing the development of monoglycolipid-specific antibodies in mice, the same as raft immunization does. The antisera obtained by injection with Vero, Karpas, and EL4 cell suspensions did not react with certain specific glycolipids, and only the antisera obtained by immunization with ACHN cell suspensions yielded a mono-specific reaction with sialylGb5 (Fig. 2). This suggests that immunization with suspensions of whole cells does not usually induce the development of monoglycolipid-specific antibodies and that the rafts on the cell surface of ACHN cells assemble in a manner that is favorable for inducing immune reactions against sialylGb5.

Next, we examined the quantitative and qualitative kinetics of the production of the specific antibodies in sera by immunization with rafts derived from ACHN cells. The antisera obtained after each immunization were examined by dot-blot immunostaining and TLC immunostaining (Fig. 3a). The relative amounts of antibodies that bound to rafts dot-blotted on a PVDF membrane or lipids separated on a TLC plate were shown as PSL (Fig. 3b). Production of IgM class anti-raft antibodies was detected after the second immunization, and it peaked after the third immunization, and then decreased. Production of IgG class anti-raft antibodies was also detected after the second immunization, but at a low level, and it continued to increase even after the fourth immunization. The specificity of the secondary antibodies used for typing the immunoglobulin class of anti-raft antibodies was confirmed in advance (data not shown). No anti-sialylGb5 antibodies were detected not after the first immunization (data not shown). They were faintly detected after the second immunization, and then increased in an immunization time-dependent manner. These results indicate that the production of anti-raft antibodies in mice is a typical immune response accompanying the class switch from IgM antibodies to IgG antibodies. Interestingly, the #3 antisera of the third immunization gave strong reactivity with the lower band glycolipid, while that of the fourth

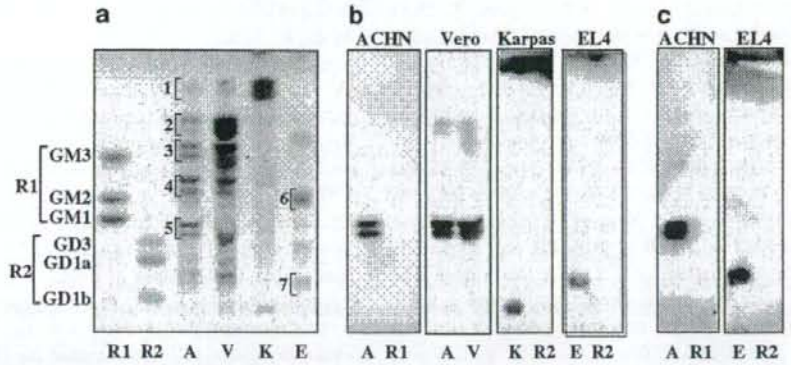

Fig. 1 TLC immunostaining with antisera against rafts components. The lipids extracted from ACHN (*A*), Vero (*V*), Karpas (*K*), EL4 (*E*) cells and the reference glycolipids (*R1* GM3, GM2, GM1; *R2* GD3, GD1a, GD1b) were separated by TLC and chemically stained with Orcinol reagent (**a**) or immunostained with antisera of Balb/c mice (**b**) and C57BL/6 mice (**c**) that had been immunized with rafts prepared from ACHN cells, Vero cells, Karpas cells and EL4 cells. Lipids extracted from 5×10^6 cells and 1×10^6 cells of each cell line are subjected to TLC for Orcinol staining and immunostaining, respectively. *1* LacCer; *2* Gb3; *3* Gb4; *4* Gb5; *5* sialylGb5; *6* GM2; *7* GD2

immunization reacted strongly with the upper band glycolipid (Fig. 3a). The result may indicate that ceramide structure is also involved in antigen presentation of glycolipid in rafts.

Next, we examined the correlation between the amounts of rafts injected and anti-raft antibody production. To do so, we immunized C57BL/6 mice with rafts prepared from various numbers of EL4 cells and evaluated the subsequent production of anti-EL4 raft antibodies by flow cytometry and TLC immunostaining. As shown in Fig. 4a, the amounts of anti-EL4 raft antibody increased with the amounts of EL4 rafts injected. Injection with the rafts prepared from 0.12×10^7 EL4 cells induced a slight elevation of reactivity, and the rafts prepared from 3×10^7 EL4 cells were sufficient to induce maximum reactivity. Rafts prepared from more than 0.6×10^7 EL4 cells appeared to be needed to obtain a significant level of anti-GD2 antibodies, (Fig. 4b).

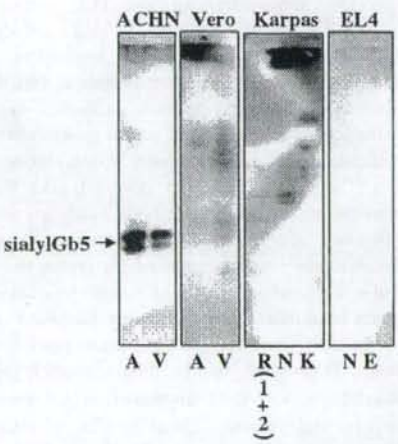

Fig. 2 TLC immunostaining with antisera against the cell suspension. The lipids were extracted from the cells as shown in the legends to Fig. 1 and from NALM-6 cells (*N*), and separated by TLC. The plates were immunostained with antisera from Balb/c mice immunized with the suspension of irradiated ACHN cells, Vero cells, Karpas cells, and EL4 cells

Since EL4 cells are derived from C57BL/6 mice, no immune responses to EL4 cells or EL4 cell components should be usually induced in syngeneic C57BL/6 mice. However, the injection of C57BL/6 mice with the EL4 rafts resulted in production of anti-raft antibodies in syngeneic mice as shown above. We therefore tried using flow cytometry to corroborate that injection of raft suspensions can induce anti-raft antibody production in syngeneic mice. The results showed that the antisera of C57BL/6 mice injected with rafts of syngeneic melanoma cell line B16F1 bound to B16F1 cells (Fig. 5a). Both mouse myeloma cell line P3U1 and lymphoma cell line RL-2 are derived from Balb/c mice, and antisera from Balb/c mice injected with rafts of these syngeneic P3U1 (Fig. 5b) and RL-2 rafts (Fig. 5c) were also confirmed to bind to P3U1 cells and RL-2 cells, respectively. Injection of mice with a PBS suspension of irradiated syngeneic cells did not result in the production of antisera that bound to syngeneic cells (data not shown).

Since repeated immunization of self- or syngeneic antigens is thought to induce autoimmune diseases, we repeated injection of C57BL/6 mice with EL4 rafts or Balb/c mice with the P3U1 rafts and investigated whether the mice produced anti-DNA antibodies by ELISA. The average A_{450} of anti-ssDNA IgM in the sera of the mice injected with PBS and the syngeneic rafts was 0.247±0.027

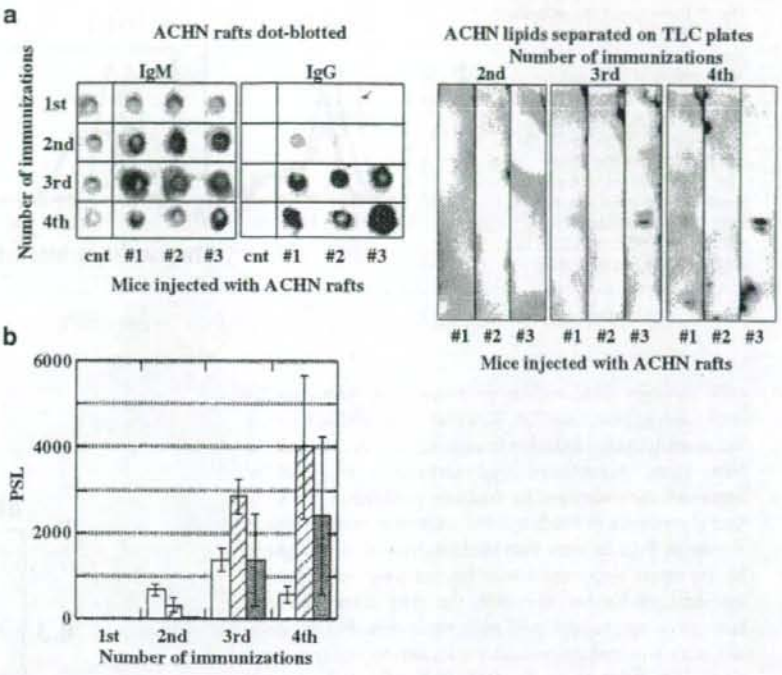

Fig. 3 Kinetics of production of antibody against ACHN rafts. Balb/c mice were injected with an ACHN raft suspension in triplicate (*#1, #2, #3*) or PBS (*cnt*) four times at 7 day intervals, and the sera were obtained 5 days after each immunization. The rafts dot-blotted on PVDF membranes were probed with each antiserum, and then probed with the HRP-conjugated anti-mouse IgM μ chain-specific antibodies or IgG γ chain-specific antibodies as secondary antibody. The lipids separated on the TLC plate were probed with each antiserum, and then with the HRP-conjugated anti-mouse IgG+M antibodies. **a** The images of dot-blot immunostaining of ACHN rafts (*left*) and TLC immunostaining of ACHN lipids (*right*) with the antisera. **b** Measurement of anti-raft IgM antibodies (*open column*), the anti-raft IgG antibodies (*striped column*), and anti-sialylGb5 antibodies (*shaded column*)

(column 1 in Fig. 6) and 0.240±0.043 (column 2 in Fig. 6), respectively, and the difference between the two groups was not significant. The A_{450} for anti-ssDNA IgM in the serum of NZB/WF1, which are well known to spontaneously develop autoimmune disease, was 0.325. No elevation of IgG class anti-DNA antibodies or anti-dsDNA antibodies was observed in the sera of either the immunized mice or NZB/WF1 mice (data not shown). No anti-DNA antibody production or other diagnostic signs of autoimmune disease were observed in these mice. These results show that the development of antibodies against syngeneic rafts components by the mice was not due to the development of an autoimmune disease.

The results of this study show that subcutaneous injection of mice with rafts prepared from specific cell lines induces production of antibodies that recognize single glycolipids, namely monoglycolipid-specific antibodies. For example, rafts prepared from ACHN cells and Vero

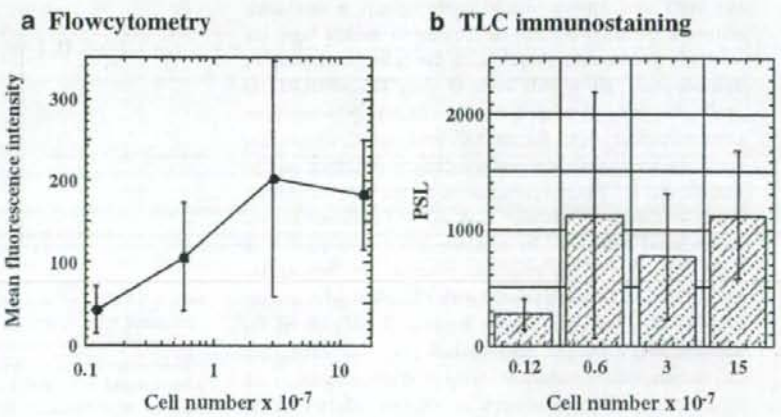

Fig. 4 Reactivity of mouse sera after immunization with the rafts prepared from various numbers of *EL4* cells. The sera were obtained from C57BL/6 mice immunized with rafts prepared from 0.12, 0.6, 3 and 15×10^7 EL4 cells. The experiments were performed in triplicate. **a** Evaluation of antibody reactivity to EL4 cells by flowcytometry. **b** Evaluation of antibody reactivity to GD2 by TLC immunostaining

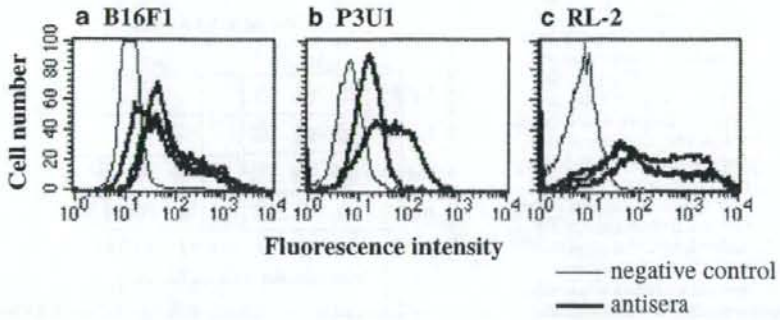

Fig. 5 Flow cytometric analysis of the antisera of mice immunized with syngeneic rafts. Cells were stained with the antisera of C57BL/6 mice immunized with B16F1 rafts (**a**), Balb/c mice immunized with P3U1 rafts (**b**) and Balb/c mice immunized with RL-2 rafts (**c**), and analyzed by flow cytometry (*bold line*). The sera of each mouse injected with PBS were used as a negative control (*thin line*)

cells strongly induced the production of mono-specific antibodies against sialyGb5. However, since sialyGb5 is not the quantitatively predominant glycolipid in ACHN cells or Vero cells, quantitative lipid dominance may not be necessary for monospecific antibody production. Since all four glycolipids to which specific antibodies were produced shown in Fig. 1b were sialylated, sialylation is thought to be the most important factor for inducing monospecific antibody production. However, the rafts from other cell lines gave no production of such antibodies. For example, although B16 melanoma cells are known to highly express GM3 [8], injection of the B16 melanoma rafts did not induce monoglycolipid-specfic antibody. Since Kawashima *et al.* [9] reported that when they intravenously injected ten strains of inbred mice with 100 μg of gangliosides adsorbed to *Salmonella minnesota*, gangliosides such as GD3, GD2, GD1b, GT1a, and GQ1b that have a trisaccharide sequence of NeuAcα2,8NeuAcα2,3Gal induced high-titer antibody responses, whereas gangliosides such as GM4, GM3, GM2, GM1, GD1a, and GT1b that have a disaccharide sequence of NeuAcα2,3Gal induced low-titer antibody responses, the diversity of immunogenicity among the glycolipids should be present. Since SSEA-4, an epitope carried by sialyGb5 has been well known highly immunogenic, a saccharide sequence of sailylGb5 can be thought to induce high-titer antibody production. Therefore, if the cells contain highly immunogenic glycolipids such as sialyGb5 and GD2 in lipid rafts, these glycolipids may be effectively presented as immunological targets for antibody production, whereas the rafts containing only low immunogenic glycolipids may be insufficient for antigen presentation to produce anti-glycolipids antibodies. Yamazaki Y. *et al.* [10] obtained several monoclonal antibodies by injecting mice with HL60 cell lipid rafts. One of the antibodies reacted with both GM1a and GD1b, and another reacted with phosphatidylglucoside. HL60 cells, however, mainly express glycolipids of the neolactoseries, not the ganglioseries [11], suggesting that raft immunization enables antibody production against such an extremely minor glycolipid. In order to induce effective

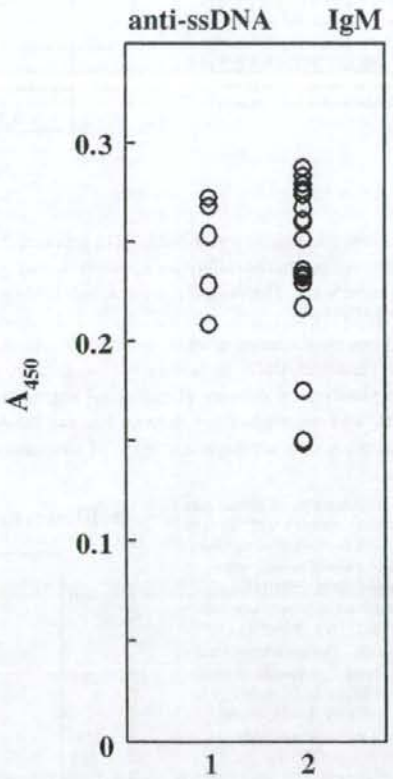

Fig. 6 ELISA of anti-ssDNA antibodies in the sera of mice immunized with syngeneic rafts. Calf thymus ss-DNA was coated and probed with the serum of C57BL/6 or Balb/c mice injected with PBS (*column 1*) and C57BL/6 mice injected with EL4 rafts or Balb/c mice injected with P3U1 rafts (*column 2*). The mean values of triplicate experiments are shown

immune responses against glycolipids in mice, a large amount of purified antigen usually must be immobilized by adsorbing it to the cell walls of bacteria, such as *Salmonella minnesota*, or by incorporating it into liposomes [12], whereas rafts themselves are insoluble and do not need to be immobilized. Furthermore, without mixing with Freund's adjuvant, rafts may retain adjuvant effects and be capable of inducing an immune response even in syngeneic mice.

It still remains unclear how monoglycolipid-specific antibodies are produced, which cells should be used for raft preparation, and to which glycolipids antibodies are predominantly produced. Although further experiments are certainly needed to answer these questions, raft immunization can be used as an effective method of producing monoclonal antibodies against glycolipids and can be applied as new approach in many fields.

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Mutated D4-guanine diphosphate–dissociation inhibitor is found in human leukemic cells and promotes leukemic cell invasion

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Objective. **Rho GTPase may be involved in human cancer invasion via the augmentation of cell motility and adhesion. We report on two point mutations of the D4-guanine diphosphate (GDP)–dissociation inhibitor (GDI) gene, one of the Rho-GDIs, which were found in a human leukemic cell line, Reh, and the mutated D4-GDI functions as an accelerator of leukemic cell invasion.**

Material and Methods. **We investigated the altered activity of GDP dissociation by mutated (mt) D4-GDI and the functions of this mt and wild-type (wt) D4-GDI in invasion. The mice inoculated with wt or mt D4-GDI vector–transfected Raji cells were observed and examined pathologically. Adhesiveness and cell motility of wt or mt D4-GDI vector–transfected Raji cells were examined. Finally, it was examined whether Rho activation was changed by mutation of D4-GDI under the condition of Rho-GDI knockdown.**

Results. **Two point mutations of the D4-GDI gene were found in Reh cells. The region of mutations is conserved among members of the Rho-GDI family at the amino acid level. D4-GDI with two mutations (V68L and V69A) functioned in a dominant negative manner in the inhibition of GDP dissociation from Rho. Severe combined immune-deficient mice inoculated with Raji cells developed hemiparalysis. The Raji cells were present in bone marrow and peripheral blood, and hepatic invasion was observed in 20% of the mice. Mice inoculated with wt D4-GDI vector–transfected Raji cells (wt D4) showed later paralysis and none developed hepatic invasion. Mice inoculated with mt D4-GDI–transfected Raji cells (mt D4) showed a 5-day reduction in the time to paraplegia and death. In addition, hepatic invasion was evident in 80% of mice transplanted with mt D4 cells. There were no differences in growth rates and amounts of guanine triphosphate (GTP)–bound Rho, cdc42, or Rac among all clones, however, GTP-bound Rho in mt D4 clone with short hairpin RNA (shRNA) vector for Rho-GDI knockdown was increased compared with wt D4 clone with shRNA vector for Rho-GDI knockdown. The mt D4 cells showed an augmentation of adhesiveness and cell motility. On the other hand, wt D4 cells showed a decreased ability of cell motility.**

Conclusion. **These results suggest the mutated D4-GDI functions as a dominant negative molecule against the wt D4-GDI and accelerates invasion via regulation of cytoskeletal machinery.**

Human leukemia progression is a process by which leukemic cells acquire more malignant properties, such as invasiveness. We previously established in vivo experimental systems of human leukemia invasion using severe combined immune-deficient (SCID) mice and reported that Rho activation augmented human leukemic cells invasion and changed the pattern of organs targeted by leukemic cells through the acceleration of leukemic cell adhesion [1].

The Rho, Rac, and Cdc 42 GTPases belong to the small guanine triphosphate (GTP)–binding protein family, a part of the Ras superfamily, and regulate various actin filament–dependent cell functions, such as cell adhesion, cell

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motility, and cytokinesis [2–5], as well as certain gene expressions [6]. These GTPases are active only in GTP-bound states and the exchange of GTP and guanine diphosphate (GDP) is strictly regulated by three types of regulatory proteins; GDP dissociation stimulators (GDS), GDP dissociation inhibitors (GDI), and GTPase activating proteins (GAP). Some GDS and GAP from the Rho family and three Rho GDIs have been isolated [7]. D4-GDI, one of the Rho GDIs, is preferentially expressed in hematopoietic cells, and Rho-GDIγ is expressed in the brain, lungs, kidneys, testes, and pancreas, while Rho-GDI is ubiquitously expressed in all mammalian organs [8–10]. Rho-GDI binds the majority of Rho-family GTPases in the cytoplasm, maintaining Rho in an inactive form in which it cannot interact with effecter targets or other regulatory proteins [11]. On the other hand, Rho-GDI also associated weakly with the GTP-bound forms of Rho, Rac, and Cdc42 [12,13]. This weak interaction resulted in an inhibition of the intrinsic and GAP-stimulated GTPase activities of the Rho GTPases. Thus, Rho-GDI appears to be a molecule capable of blocking the GTP binding/GTPase cycle at two points: at the GDP–GTP exchange step and the GTP hydrolytic step. Further studies demonstrated that Rho-GDI associates with a Rho-GDI displacement factor from the ERM family, which consists of ezrin, radixin, and moesin. ERM interacts with both an adhesion molecule—CD44—and F-actin, resulting in association of the actin cytoskeleton with the plasma membrane [14]. D4-GDI has been identified as a Rho-GDI–like protein that is approximately 68% homologous with Rho-GDI, and is preferentially expressed at very high levels in hematopoietic cells, including erythroid, granulocytic, monocytic, and lymphoid cells [8]. In another report, expression of D4-GDI in lymphocytes was emphasized and D4-GDI was named Ly-GDI [9]. The inhibitory effect of D4-GDI on GDP dissociation was specific for Rho, but not Ras or Rap [8]. Like other Rho-GDIs, D4-GDI was postulated to bind and inhibit Rho GTPases. However, much yet remains to characterize the specificity of D4-GDI [15,16].

D4-GDI has been reported to be a substrate of the apoptosis protease CPP32. D4-GDI was rapidly truncated to a 23-kDa fragment in Jurkat cells with kinetics that parallel the onset of apoptosis following Fas cross-linking with agonistic antibody or treatment with staurosporine [17]. Furthermore, Krieser et al. [18] showed that a cleaved 26-kDa fragment derived from D4-GDI resided in the cytoplasm of undamaged cells, whereas after cleavage by CPP32, the 22-kDa form of D4-GDI translocated to the nucleus [18]. These lines of evidence suggest that D4-GDI is involved in cell-shape alterations and/or changes in cell fragmentation during leukocyte apoptosis.

A number of Ras gene mutations have been found in a wide variety of human malignant tumors, including leukemias and lymphomas [19]. Point mutations in Ras cause decreased GTPase activity and may transform in some leukemic cells. Rho, a member of the Ras family, has not been associated with transformation, and no Rho mutations have been detected in human malignant tumors to date [20]. However, it has been reported that some regulatory proteins for Rho GTPases, dbl, tiam1, and vav, are reportedly associated with tumor development [7,21,22]. The Dbl oncogene was originally discovered because of its ability to induce focus formation and tumorigenicity when expressed in NIH-3T3 cells [23]. Tiam, however, was first identified as an invasion-inducing gene using proviral tagging in combination with in vitro selection for invasiveness [24]. Furthermore, the Rho family of small GTPases, including Rac, Cdc42, and Rho, has been implicated in the regulation of many aspects of cancer cell motility and invasion, including cell polarity, cytoskeletal organization, and transduction of signals from the extracellular environment [25–28].

In this study, we identified two point mutations of the D4-GDI gene in a human B-cell leukemia cell line, Reh, and analyzed the functions of the mutated (mt) D4-GDI in vitro and in vivo employing an experimental system consisting of human leukemic cell invasion in SCID mice.

Materials and methods

Human leukemic cells and cell culture

Two acute lymphoblastic leukemia cell lines (Reh and HPB-ALL) and three Burkitt's lymphoma cell lines (Raji [ATCC, CCL-86], Ramos [ATCC, CRL-1923], and Daudi [ATCC, CCL-213]) were examined. The Reh cell line was established from a girl with a common form of acute lymphoblastic leukemia [29]. This cell line is known to be accompanied by the TEL-AML1 fusion gene due to chromosomal translocation [30]. We used reverse transcriptase polymerase chain reaction (RT-PCR) to confirm that our Reh cells expressed mRNA derived from the TEL-AML1 fusion gene (data not shown). The Raji cell line was derived from Burkitt's lymphoma. The HPB-ALL cell line was derived from a pediatric T-cell leukemia [31]. Cells were cultured in the presence of 5% CO_2 at 37°C using RPMI-1640 medium supplemented with 10% fetal bovine serum. Normal human peripheral blood lymphocytes from healthy Japanese men were also examined with informed consent.

RT-PCR and DNA sequencing

Total RNA was extracted from each sample (5–10 × 10^6 cells) using ISOGEN (Nippon Gene, Toyama, Japan). RNA was reverse-transcribed into first strand cDNA using a First-Strand cDNA Synthesis Kit (Amersham-Pharmacia Biotech, Buckinghamshire, UK). D4-GDI cDNA was isolated by PCR amplification from first-strand cDNA using the N-terminal primer (5′-TAAATA GATCAGAATGACTGAA-3′) and the C-terminal primer (5′-AGAATTCTTCCA AGGTGGCAA-3′). PCR was performed in 10 mM Tris-HCl (pH 9.0), 2.0 mM $MgCl_2$, 50 mM KCl, 0.2 mM each deoxyribonucleoside triphosphate, and 0.5 μM each PCR primer using Taq DNA Polymerase (Toyobo, Tokyo, Japan). Thirty cycles were run with denaturation at 94°C for 60 seconds, annealing at 55°C for 60 seconds, and extension at 72°C for 60

seconds. RT-PCR products were cloned into a pGEM-T vector (Promega, Madison, WI, USA), and analyzed with a Thermo Sequenase fluorescent–labeled primer cycle sequencing kit (Amersham-Pharmacia Biotech) using T7 and Sp6 fluorescent primer and a DNA sequencer (MegaBase 1000, Molecular Dynamics, Sunnyvale, CA, USA). The fluorescent primers used for sequencing were forward, 5′-GTCGCAGGAA ATGGACA AAGAT-3′ and reverse 5′-TCCAGTAA GGTCCATG GTGATT-3′.

Sequences of genomic D4-GDI DNA

DNA was prepared from Reh cells by standard methods with sodium dodecyl sulfate–proteinase K [32]. A portion of the D4-GDI gene that included the mutations was amplified using the N-terminal primer (5′-CACCACAGAAGTCCCTGAAAGA-3′) and the C-terminal primer (5′-TCCA GTAAGGTCCATGGT GATT-3′). PCR products were cloned into a pGEM-T vector and sequenced. After partial sequencing of the D4-GDI intron (data not shown), PCR products were analyzed by direct sequencing methods using the fluorescent forward primer (5′-CACCCAC TATACACATGTCTCT-3′) for the D4-GDI gene intron. Reh cells were also obtained from other laboratories and the D4-GDI gene was sequenced by the following method in order to eliminate any contamination of cells and to confirm the mutations. RT-PCR was performed with another N-terminal primer (5′-ACAGA GACGTGAAGCACTGAA-3′) and C-terminal primer (5′-GATG CATCAA TAAGGAAATGT-3′). These primers flanked the initial primers and were used to exclude contamination of PCR products and plasmids. PCR products were analyzed by direct sequencing method.

Construction of mt and wild-type D4-GDI expression vectors and short hairpin RNA vector for knockdown of Rho-GDI–α

Mutated D4-GDI cDNA of Reh cells was generated by RT-PCR. Wild-type (wt) D4-GDI cDNA was generated from HPB-ALL cells by RT-PCR. Vectors containing wt or mt D4-GDI cDNA with a myc-tag driven by the SRα promoter were constructed. This vector contained the neomycin-resistance (neo^r) gene driven by the SV40 promoter. The specific sequences for Rho-GDI small interfering RNA were searched by siDirect online software (RNAi Corporation, Tokyo, Japan). As a result, nucleotide number of human Rho-GDI–α 1191–1213 (3′UTR TCGGTCCCGTCTAAC CATGATGC) as Rho-GDI–α and scramble 23-nucleotide as control were generated. DNA-based small interfering RNA vectors were constructed in pBLOCK-iT6 DEST vector (Invitrogen, Carlsbad, CA, USA) for short hairpin RNA (shRNA) synthesis.

Transfection of wt or mt D4-GDI gene and shRNA vector for Rho-GDI knockdown into Raji cells

Wild-type or mt D4-GDI expression vector or shRNA vector for Rho-GDI knockdown was transfected into Raji cells by electroporation using a Gene Pulser (Bio-Rad, Hercules, CA, USA). The SRα–myc-tag vector or shRNA vector with scramble 23-nucleotide was transfected into Raji cells as a control. The Raji cells were cultured in culture media with G418 (800 μg/mL; Sigma-Aldrich, Tokyo, Japan) or blasticidin (10 μg/mL; Invitrogen, Carlsbad, CA, USA) for 14 days, followed by subcloning in a 96-well plate twice. Expression of D4-GDI or Rho-GDI–α protein was confirmed by Western blotting using a rabbit anti–D4-GDI or Rho-GDI polyclonal antibody (Zymed Laboratory, San Francisco, CA, USA). Blotted membranes were treated with peroxidase-conjugated anti-rabbit immunoglobulin antibody and visualized with electrochemiluminescence (Amersham-Pharmacia Biotech). The protein concentration was measured by BCA protein assay reagent (Pierce, Rockford, IL, USA).

Transplantation of leukemic cells into SCID mice

SCID mice (C.B.17 SCID mice, female, 7 to 9 weeks after birth; Clea, Tokyo, Japan) were maintained under specific pathogen-free conditions, and 2×10^7 Raji cells with/without wt or mt D4-GDI or Rho-GDI shRNA were suspended in 100 μL culture medium and injected into the tail veins of mice.

Analysis of leukemic cell invasion in SCID mice

Development of hemiparalysis in the mice was defined as the state in which they showed no motion of their hemilateral lower extremities. On day 17 or 20, when all mice were still alive and some showed hemiparalysis, the mice were sacrificed. Peripheral blood was prepared from the orbital vein plexus and cells were taken from the bilateral femurs and tibiae, and the spleen. The peripheral blood was subjected to hemolysis before being washed in phosphate-buffered saline (pH 7.4). Samples were then subjected to staining with anti-human CD19 monoclonal antibody (phycoerythrin-conjugated; DAKO, Glostrup, Denmark, diluted 1:100) for analysis with a flow cytometer (EPICS XL-MCL; Beckman Coulter, Hialeah, FL, USA). The systemic organs of mice were also prepared for pathological analysis by fixation in 10% formaldehyde in phosphate-buffered saline, embedding in paraffin, sectioned and then stained with hematoxylin-eosin. Immunohistochemical analyses were performed with anti-human CD19 monoclonal antibody and anti-proliferating cell nuclear antigen (PCNA) monoclonal antibody (Oncogene Science, Uniondale, NY, USA, diluted 1:50).

In vitro and in vivo proliferation assay

Proliferation rates of Raji cells with mt or wt D4-GDI or myc-tag only and shRNA vector for Rho-GDI knockdown or scramble 23-nucleotide were determined using the MTT method. These three clones were placed in eight wells of a round-bottomed 96-well plate at a concentration of 2×10^3 cells/100 μΛ/well and cultured for 48 hours, followed by addition of 5 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) at a concentration of 10 μL/well and further cultured for 4 hours. After the cells had settled on the plate, 100 μL 0.04 N HCl plus isopropanol was added. The resultant mixture was stirred and then measured using an enzyme-linked immunosorbent assay reader (Microplate Reader Model 450; Bio-Rad) for absorbance at 570 nm and 630 nm.

The in vivo proliferative capabilities of leukemic cells were investigated by the PCNA labeling index in situ [33]. The number of nuclear PCNA-positive cells and total cells in the vertebrae were counted in 10 fields.

Cell motility assay

Cell migration ability was assessed in 48-well chambers using polyvinylpyrrolidone-free polycarbonate membranes with 5-μm or 3-μm pores (NeuroProbe, Inc., Gaithersburg, MD, USA). RPMI-1640 supplemented with 1% pasteurized human plasma was placed in lower wells, and used to dilute the cells in upper wells. After 3 hours at 37°C, the membrane was removed, washed on the upper side with phosphate-buffered saline, then fixed and stained with DiffQuik (NeuroProbe). All assays were done in triplicate, and migrated cells were counted in five randomly selected