fields at 600-fold magnification. General and spontaneous migration was determined in the absence of chemokines.

Adhesion assays

Adhesion of Raji cells to the extracellular matrix or cells was assessed. Extracellular matrices (Matrigel; Becton-Dickinson, Mountain View, CA, USA), human fibronectin, laminin, and collagen type IV (Asahi Techno Glass, Funabashi, Chiba, Japan) were used in the 24-well Biocoat cellware (Becton-Dickinson). Human bone marrow stromal cells, which were obtained from bone marrow specimens of nonhematological patients, with informed consent, were seeded in 24-well plates prior to 24-hour adhesion assays. Cells were fluorescently labeled with 2 μM 2,7-bis-(2-carboxyethyl)-5 (and 6) carboxyfluorescein (BCECF; Molecular Probes, Eugene, OR, USA) for 30 minutes at 37°C. Labeled cells were washed twice, resuspended with RPMI to achieve a concentration of 2×10^5 cells/mL, and added to each well. After incubation with fixative, plates were washed and the number of fluorescent cells bound was determined by proportionality to the remaining BCECF fluorescence measured using a FluorImager 595 (Molecular Dynamics).

Affinity-precipitation of cellular GTP-bound Rho, Cdc42, and Rac

Ren et al. [34] developed a method based on evidence that Rho effectors interact only with GTP-bound Rho for the measurement of Rho activity [34]. Binding of Rho to the Rho-binding domain (RBD) from the effector protein Rhotekin inhibited both the intrinsic and GAP-enhanced GTPase activity of Rho [35]. Therefore, Rhotekin RBD was used to affinity-precipitate cellular GTP-Rho. Cells were washed with ice-cold Tris-buffered saline and lysed in RIPA buffer (50 mM Tris [pH 7.2], 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 500 mM NaCl, 10 mM $MgCl_2$, 10 mg/mL each of leupeptin and aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were clarified by centrifugation at 13,000*g* at 4°C for 10 minutes, and equal volumes of lysates were incubated with GST-RBD (a fusion of RBD with glutathione S-transferase, 20 μg) beads at 4°C for 45 minutes. Beads were washed four times with buffer B (Tris buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 10 mg/mL each of leupeptin and aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride). Bound Rho proteins were detected by Western blotting using an anti-RhoA monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Densitometric analysis was performed using NIH image version 1.62. The amount of RBD-bound Rho was normalized to the total amount of Rho in cell lysates for the comparison of Rho activity (level of GTP-bound Rho) in different samples. Depending on cell conditions and types, and different batches of GST-RBD, the RBD-bound Rho accounts for ~0.5% to 5% of total Rho. The measurement of Rac activity was performed using the Rac Activation Assay kit (Cytoskeleton, Denver, CO, USA) according to the manufacturer's protocol.

[^{3}H]GDP dissociation assay

D4-GDI (wt, V68L, V69A, and both V68L and V69A mutations) protein was synthesized using Baculo-viral expression system with Bac-to-Bac HT vector (Invitrogen, Carlsbad, CA, USA) and Sf9 cells. His-tag D4-GDI proteins in cell lysates were purified using Ni-NTA agarose and ProBond Purification system (Invitrogen) according to the manufacturer's protocol. The inhibitory activities of wt, mt (V68L, V69A, and both V68L and V69A mutations), and wt plus each mt D4-GDI on GDP dissociation from isoprenylated Rho were determined using a filtration assay, as described previously by Chuang et al. [13]

Statistical analysis

All results were evaluated using Student's *t*-test–based statistics. Experiments were performed at least three times each.

Results

Detection of mutations of D4-GDI cDNA or genomic DNA in human leukemic cells

Results of DNA sequence analysis of the D4-GDI cDNA from the human leukemic cell lines are shown in Figure 1. Two point mutations at positions 276 (a G to C change) and 280 (T to C) were found in the D4-GDI cDNA of the Reh cell line. No mutations of the D4-GDI gene were detected in the D4-GDI genes of HPB-ALL, Raji, Ramos, and Daudi cell lines or in normal human peripheral blood lymphocytes (data not shown). Direct sequencing analysis of genomic DNA showed that these mutations were present on one allele (Fig. 2). These two point mutations of D4-GDI in the Reh cell led to conversions of valine 68 to leucine and valine 69 to alanine. The alignment of the predicted amino acid sequences of mt D4-GDI, wt D4-GDI, and other Rho-GDI family genes are shown in Figure 1. These two mutations exist in the partially conserved region at the amino acid level.

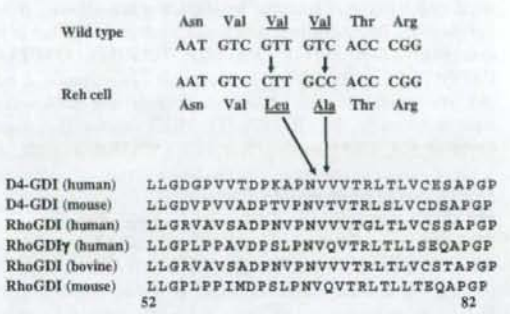

Figure 1. Two point mutations of D4-GDI cDNA in Reh cells and alignment of the predicted amino acid sequence. Two point mutations in D4-GDI were detected in Reh cells (arrows). These changes resulted in a guanine to cytosine substitution at position 276 and a thymine to cytosine substitution at nucleotide 280 (underlined). The alignment of predicted amino acid sequences of D4-GDI and Rho-GDI family genes. The two D4-GDI point mutations in Reh cells led to a valine 68 to leucine change and a valine 69 to alanine change. This region is highly conserved in Rho-GDI family members.

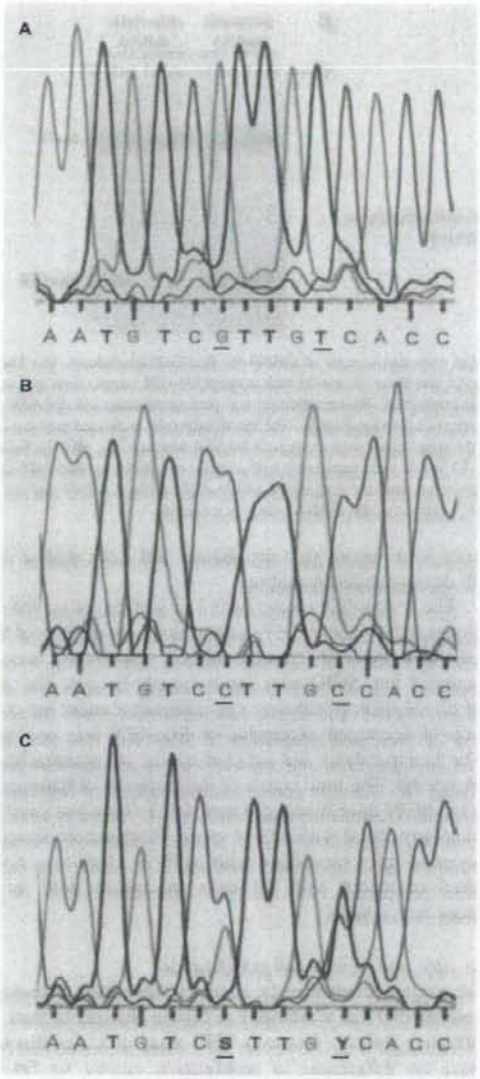

Figure 2. Heterozygous mutations of the D4-GDI gene in Reh cells. (**A**) Normal D4-GDI sequence from normal human peripheral blood lymphocytes. (**B**) Two point mutations in D4-GDI in Reh cells (underlined). These changes resulted in a guanine to cytosine substitution at position 276 and a thymine to cytosine substitution at nucleotide 280. (**C**) Direct sequence analysis of DNA amplified from genomic DNA of Reh cells showed identical mutations in one allele (heterozygous). Arrowheads indicate the two peaks, guanine and cytosine at position 276, and thymine and cytosine at nucleotide 280.

Gene transfer of wt or mt D4-GDI expression vector into Raji cells

A human leukemic cell line (Raji) was used in order to clarify the functions of mt or wt D4-GDI, because there were no mutations of D4-GDI gene in the Raji cells. Some clones with wt D4-GDI, mt D4-GDI, or the myc-tag vector were obtained and used in subsequent experiments. Expression of exogenous D4-GDI in these clones was confirmed by Western blotting using anti–D4-GDI polyclonal antibody. These cells expressed a protein of approximately 29 kDa, which was recognized by the D4-GDI antibody (Fig. 3A). The lower bands were endogenous D4-GDI in Raji cells and the upper bands were the exogenous D4-GDI with the myc-tag. We detected the expression of exogenous D4-GDI protein by Western blotting using anti-myc-tag antibody (9E10) (data not shown). The amounts of exogenous D4-GDI were almost the same as the endogenous D4-GDI in clones 21 and 26 with wt D4-GDI and in clone 13 with mt D4-GDI.

Gene transfer of a shRNA vector for Rho-GDI knockdown into Raji cell clones with wt or mt D4-GDI expression vector

The Raji cells with the wt D4-GDI (clone 3) or the mt D4-GDI (clone 13) were transferred with a shRNA vector for Rho-GDI knockdown or a vector of scramble shRNA as a control. Clones that were selected by blasticidin were examined by Western blotting using anti–Rho-GDI polyclonal antibody. Expression of Rho-GDI in clone wt3-21 and mt13-27 with Rho-GDI shRNA vector was decreased compared with clone wt3-1 and mt13-3 with scramble shRNA vector as a control (Fig. 3B). The clones with decreased expression under a quarter of Rho-GDI were established from the Raji cells with the wt D4-GDI, on the other hand, clones with a decreased expression under a half of Rho-GDI were not obtained from the Raji cells with the mt D4-GDI.

Exogenous D4-GDI expressions alter invasion of human leukemic cells in SCID mice

Wild-type D4-GDI clones (21 and 26), mt D4-GDI clones (13 and 20), and two myc-tag clones (1 and 4) were inoculated into SCID mice intravenously. The hemiparalysis and survival curves are summarized in Figure 4A. All mice (n = 18) inoculated with the myc-tag clone (as a control) developed hemiparalysis at 18 to 41 days (mean: 24 days) after transplantation. Histological analysis of systemic organs on day 20 revealed that the myc-tag clones invaded the liver in two of the nine mice (22%). Myc-tag clones were present in peripheral blood (3–52% of white blood cells) and bone marrow (3–66% of mononuclear cells) on day 20. Myc-tag clones infiltrated both ovaries, as well. There were no invasions of myc-tag clones into the brain, salivary glands, lungs, kidneys, digestive tract, heart, adrenal glands, spleen, or thymus. In the mice with hemiparalysis, numerous monotonous blasts occupied the bone marrow of vertebrae and femora, and also extended beyond the bone into the epidural space of the spinal cord, and into neighboring muscles (Fig. 5A). The murine hematopoiesis in bone marrow was markedly suppressed

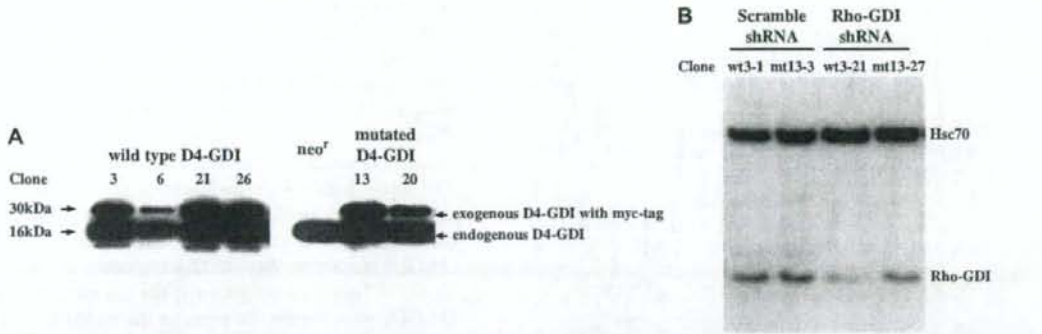

Figure 3. Gene transfer of wild-type or mutated D4-GDI expression vector into Raji cells and a vector of shRNA for Rho-GDI knockdown. (A) The Raji cells carrying the D4-GDI gene (clones 3, 6, 21, and 26 with wild-type D4-GDI vector and clone 13 and 20 with mutated D4-GDI vector) were prepared in Laemmeli's buffer and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Western blotting was performed using anti-D4-GDI polyclonal antibody, peroxidase-conjugated secondary antibody, and electrochemiluminescence for visualization. The transfected cells expressed proteins of approximately 29 kDa and 30 kDa. The 29-kDa bands were endogenous D4-GDI and the upper bands were exogenous D4-GDI with myc tag. (B) The Raji cells with the wild-type (wt) D4-GDI (clone 3, wt3) or the mutated (mt) D4-GDI (clone 13, mt13) were transferred with a vector of shRNA for Rho-GDI knockdown. Selected clones were examined by Western blotting using anti-Rho-GDI polyclonal antibody. Expression of Rho-GDI in clone wt3-21 and mt13-27 with Rho-GDI shRNA vector was decreased compared with clone wt3-1 and mt13-3 with scramble shRNA vector as a control.

by infiltration of human leukemic cells. In addition, the spinal cord showed spongiosis, suggesting that development of hemiparalysis in these mice was attributable to invasion of the epidural space by leukemic cells (Fig. 5A). All mice inoculated with myc-tag clones died at 20 to 43 days posttransplantation.

Hemiparalysis in SCID mice inoculated with wt D4-GDI clones was apparently delayed as compared with the mice inoculated with myc-tag clones. These mice developed hemiparalysis (Fig. 4A). Eighteen of the 26 had hemiparalysis at 18 to 49 days (mean: 33.9 days, $p < 0.01$) after transplantation. Some of the SCID mice inoculated with wt D4-GDI clones developed hemiparalysis during the 60-day observation period (69% of all mice). The remaining mice survived more than 60 days (31% of all mice). There were no invasions of Raji cells with wt D4-GDI overexpression in the liver on day 20 after transplantation (0 of the 6 mice). The wt D4-GDI clones were present in peripheral blood (3–9% of white blood cells) and bone marrow (4–10% of white blood cells) on day 20.

On the other hand, SCID mice inoculated with mt D4-GDI clones began to develop hemiparalysis earlier, i.e., on day 14, after transplantation as compared with the mice inoculated with myc-tag clones, which developed similar paralysis after day 18 posttransplantation (Fig. 4A). All mice with mt D4-GDI (n = 20) developed hemiparalysis at 14 to 20 days (mean: 17.4 days, $p < 0.01$) after transplantation.

Histological analysis of mice inoculated with the mt D4-GDI clones revealed hepatic invasion of leukemic cells on day 17 in 8 of the 10 mice (80%), and all had larger invasive areas than the control myc-tag mice (Fig. 5B and C). Furthermore, invasions of Raji cells into the brain, kidneys, and ovaries in some of the mt D4-GDI clone–transplanted mice were found (date not shown). All mice died at 17 to 26 days posttransplantation.

The wt D4-GDI clones (wt3-1 or wt3-21) or mt D4-GDI clones (mt13-3 or mt3-27) with a vector for shRNA of Rho-GDI or a vector of scramble shRNA, respectively, were inoculated into SCID mice intravenously because the effect of mt D4-GDI in leukemic cell infiltration under the condition of decreased expression of Rho-GDI was examined. The hemiparalysis and survival curves are summarized in Figure 4B. The time course of development of hemiparalysis in SCID mice inoculated with wt3-1 clone was similar to mice inoculated with wt3-21 clone. Furthermore, hemiparalysis of mice inoculated with mt13-27 clone was not altered compared with the mice inoculated with mt13-3 clone as a control.

In vitro and in vivo cell proliferation

We examined whether the altered invasiveness of leukemic cells in vivo was attributable to certain changes in their proliferative abilities. However, MTT assay indicated that there were no differences in proliferation among wt D4-GDI clones, mt D4-GDI clones, and myc-tag clones in vitro (Fig. 6). An immunohistochemical analysis using anti-PCNA antibody was done in order to identify the proportion of in situ leukemic cells in S phase. PCNA is expressed in the nuclei of cells in the S phase in parallel with incorporation of bromodeoxyuridine or [^{3}H]-thymidine [1,33]. Almost all PCNA-positive cells in the bone marrow were human leukemic cells. There were no significant differences among the PCNA-labeling indices of wt D4-GDI clones, mt D4-GDI clones, and myc-tag clones (data not shown). These results indicate that neither wt nor mt D4-GDI expression altered leukemic cell invasion via induction of cell proliferation. The proliferating activity of the wt

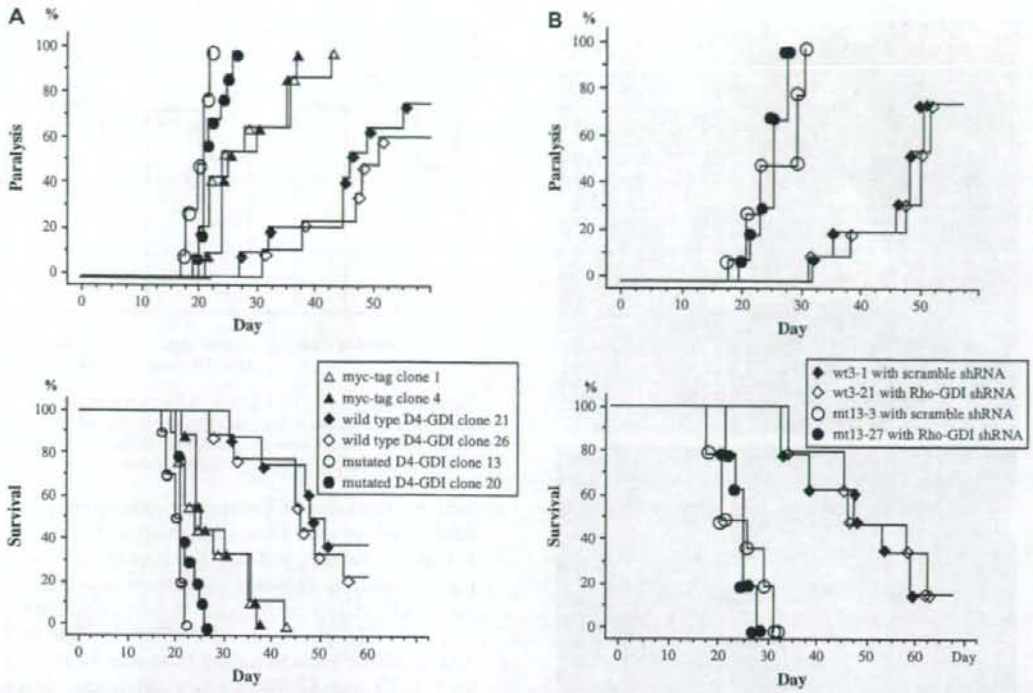

Figure 4. Alteration of hemiparalysis and survival with exogenous D4-GDI expressions in leukemic cells. (**A**) Hemiparalysis was observed in severe combined immune deficient (SCID) mice inoculated with wild-type D4-GDI clones (21 and 26), mutated D4-GDI clones (13 and 20) and myc-tag clones (1 and 4). The mice inoculated with wild-type D4-GDI clones developed hemiparalysis later than myc-tag clone–transplanted mice. On the other hand, the mutated D4-GDI clone–transplanted mice developed hemiparalysis earlier than the myc-tag clone–transplanted mice. Data on the appearance of hemiparalysis; Myc-tag clone transplanted mice (n = 18) at 18 to 41 days (mean: 24 days), wild-type D4-GDI–transplanted mice (n = 26) at 18 to 49 days (mean: 33.9 days, $p < 0.01$), and mutated D4-GDI–transplanted mice (n = 20) at 14 to 20 days (mean: 17.4 days, $p < 0.01$) after transplantation. (**B**) The wild-type D4-GDI clones (wt3-1 or wt3-21) or mutated D4-GDI clones (mt13-3 or mt3-27) with a vector for shRNA of Rho-GDI or a vector of scramble shRNA were inoculated into SCID mice. The hemiparalysis and survival curves are not altered. The time course of development of hemiparalysis in SCID mice inoculated with wt3-1 clone was similar to mice inoculated with wt3-21 clone. The hemiparalysis of mice inoculated with mt13-27 clone was not altered compared with the mice inoculated with mt13-3 clone as a control.

D4-GDI clones (wt3-1 or wt3-21) or mt D4-GDI clones (mt13-3 or mt3-27) with a vector for shRNA of Rho-GDI or a vector of scramble shRNA were examined using MTT assay in order to observe the function of mt D4-GDI under the condition of decreased expression of Rho-GDI. As a result, no differences between these clones (wt3-1, wt3-21, mt13-3 or mt3-27) were revealed (data not shown).

Alteration of cellular motile activity by exogenous D4-GDI expression

We attempted to estimate changes in general cell motility in response to exogenous D4-GDI expression in leukemic cells, because we did not detect differences in cell proliferation of wt or mt D4-GDI clones in vitro or in vivo. As a result, mt D4-GDI clones had increased cell motilities ($p < 0.001$), and wt D4-GDI clones had decreased cell motilities ($p < 0.01$) as compared with the myc-tag clones, without chemokines in vitro (Fig. 7). The cellular motile activities of each clone would correspond to the invasive activities (see Figs. 4 and 5). Mutated D4-GDI clone 13 showed the highest motile activity in vitro and the highest invasive activity in vivo. The motile activity of wt or mt D4-GDI clones (wt3-1, wt3-21, mt13-3, or mt3-27) with a vector of scramble shRNA or a vector for shRNA of Rho-GDI were examined in order to analyze the function of mt D4-GDI under the condition of decreased expression of Rho-GDI. As a result, no alterations between clone wt3-1 and wt3-21, or between clone mt13-3 and mt3-27, were observed (data not shown).

Augmentation of cell adhesion by mt D4-GDI expression

Cell motility consists of multiple and complex steps, including a response against chemotactic factors, cytoskeletal organization, and cell adhesion. We investigated whether exogenous D4-GDI expression altered the motile activity of leukemic cells through changes in cell adhesion. Adhesion of Raji cells, which contain mt or wt D4-GDI or the

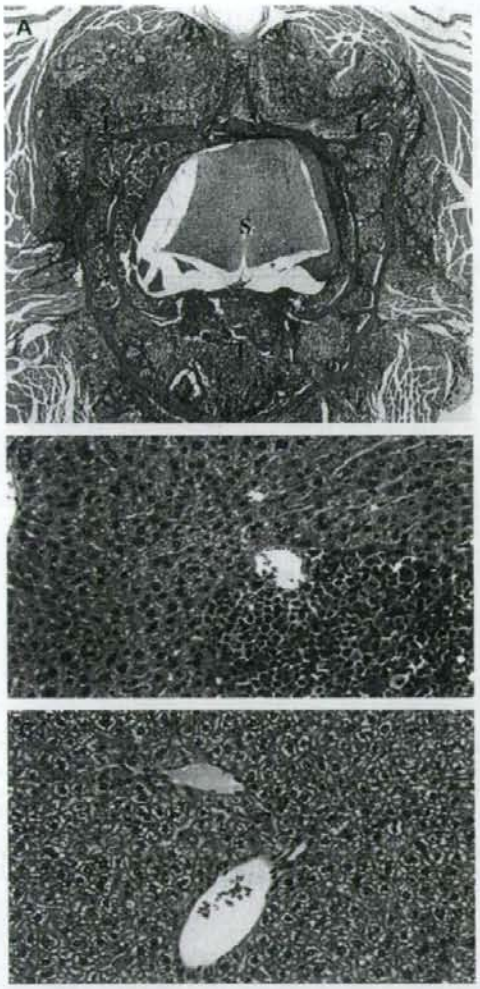

Figure 5. Raji cell invasion in severe combined immune deficient (SCID) mice. (**A**) The vertebra of an SCID mouse inoculated with myc-tag clone 1 is shown in a transverse section (hematoxylin-eosin staining). Raji cells have invaded the bone marrow, surrounding tissues, and the epidural space (day 17 after transplantation). The result is spongiosis, due to compression myelopathy of the spinal cord. S and L indicate the spinal cord and human leukemic (Raji) cells, respectively. Original magnification ×100. (**B**) Hepatic invasion by mutated D4-GDI clone 13 is shown. Original magnification ×120. (**C**) On other hand, there was no hepatic invasion in mice inoculated with wild-type D4-GDI clone 26. Original magnification ×120.

myc-tag only, to the extracellular matrix or cells was assessed as described previously [1]. Extracellular matrices (Matrigel [Becton-Dickinson], human fibronectin, laminin, and collagen type IV) were placed in 24-well dishes. In order to quantitate cell-adhesion activity, the leukemic cells were labeled with 2 μM BCECF, washed, and the fluorescence was then measured with a FluorImager 595. As a result, no significant differences in adhesiveness between these clones on each extracellular matrix (Matrigel, human fibronectin, laminin, and collagen type IV) were observed (data not shown). However, differences in adhesiveness to the stromal cells were obvious. In contrast to the myc-tag and wt D4-GDI clones, the mt D4-GDI clones showed augmented adhesiveness to human bone marrow stromal cells ($p < 0.05$) (Fig. 8). There was a significant difference in adhesion to human stromal cells between the myc-tag clones and the wt D4-GDI clones.

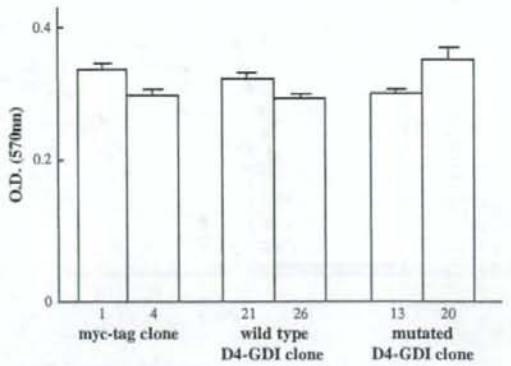

Figure 6. Exogenous D4-GDI expression does not change cell growth rates. The in vitro growth rates of myc-tag clones, wild-type D4-GDI clones, and mutated D4-GDI clones were compared by performing MTT assays. There were no significant differences in proliferative ability among these clones.

Cell-adhesion activity of wt or mt D4-GDI clones (wt3-1, wt3-21, mt13-3, or mt3-27) with a vector of scramble shRNA or a vector for shRNA of Rho-GDI was examined in order to analyze the function of mt D4-GDI under the condition of decreased expression of Rho-GDI. As a result, no differences in adhesion activity between clone wt3-1 and wt3-21, or between clone mt13-3 and mt3-27, were revealed (data not shown).

Detection of cellular GTP-bound Rho, Cdc42, and Rac in leukemic cells

We investigated whether D4-GDI (wt or mt) overexpression in leukemic cells altered Rho and Rac activity. We employed a pull-down assay using RBD affinity-precipitation and Western blotting with anti-RhoA antibody (see Materials and Methods). The proportions of activated Rho (GTP-bound Rho/total Rho) did not differ among myc-tag, wt D4-GDI, and mt D4-GDI clones (Fig. 9). GTP-bound Rho accounted for 1.3% to 1.6 % of total Rho in all experiments. In order to confirm the absence of differences in Rho activity among these clones, the immunoprecipitation with anti-RhoA antibody was carried out after metabolic pulse chase labeling with [^{35}S]-GTPγs. The uptakes of [^{35}S]-GTPγs into RhoA for 8 hours in myc-tag, wt D4-GDI, and mt D4-GDI clones were not different (data not shown). Furthermore, no differences of the

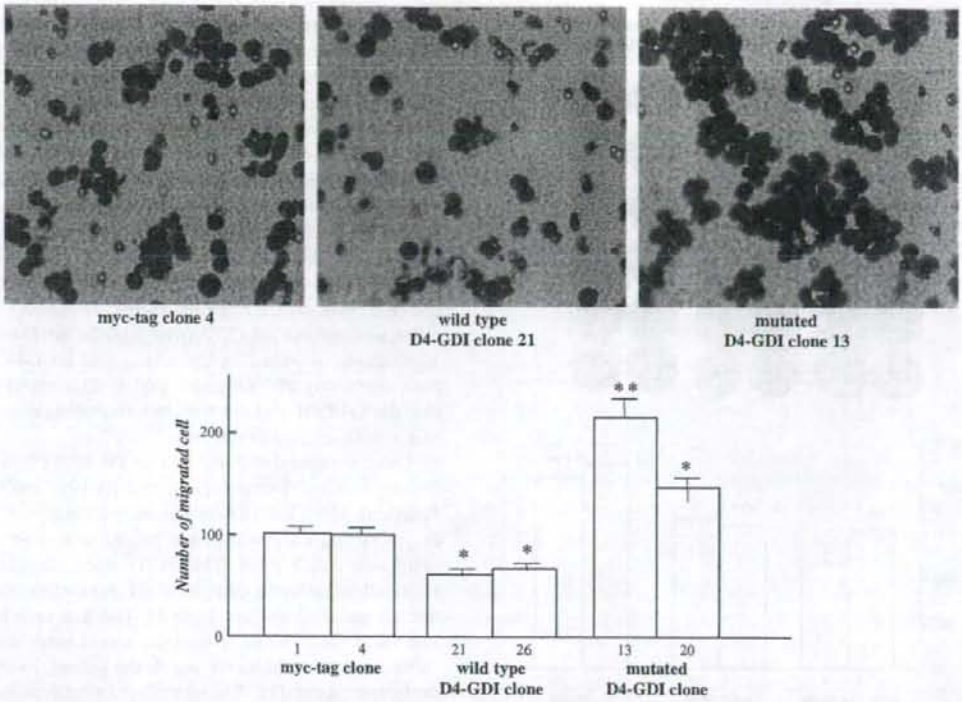

Figure 7. Alteration of cell motility by wild-type or mutated D4-GDI overexpression. The in vitro cellular motile activities of myc-tag clones, wild-type D4-GDI clones, and mutated D4-GDI clones were compared using a chemotaxis chamber (NueroProbe), without hemotactic factors (see Materials and Methods). In contrast to myc-tag clones 1 and 4, mutated D4-GDI clones 13 and 20 showed markedly increased motilities. On the other hand, wild-type D4-GDI clones 21 and 26 showed significantly decreased cell motilities. The upper colored figures show results representative of migrated leukemic cells stained with Diff-Quik (NeuroProbe). Data are represented as mean values. Error bars show standard error of mean. ** and * indicate statistically significant increases ($p < 0.001$ and $p < 0.01$, respectively) as compared to data from myc-tag clone 1 or 4.

Cdc42 and Rac activities between these clones were also observed (Fig. 9A).

Alterations of cellular GTP bound-Rho under the condition of decreased expression of Rho-GDI in leukemic cells with wt D4-GDI or mt D4-GDI overexpression were examined. The pull-down assay for Rho using the wt or mt D4-GDI clones (wt3-1, wt3-21, mt13-3, or mt3-27) with a vector of scramble shRNA or a vector for shRNA of Rho-GDI were done. As a result, the GTP-bound Rho in Rho-GDI knockdown clones (both wt3-21 and mt13-27) was slightly increased compared with clones with scramble shRNA vector (wt3-1 and mt13-3). Especially the difference of GTP-bound Rho/total Rho ratio between mt13-3 and mt13-27 was greater than the difference between wt3-1 and wt3-21. This dissimilarity between wt D4 clones and mt D4 clones in Rho activation by Rho-GDI knockdown may show that the mt D4-GDI proteins impair certain D4-GDI functions.

These results show the exogenous mt D4-GDI expression to be involved in the invasiveness of human leukemic cells through augmentation of cell motility and/or cell-adhesion activity. The altered phenotypes of leukemic cells may be caused by Rho activation due to the mt D4-GDI expression.

Mutated D4-GDI functioned
in a dominant negative manner
in the inhibition of GDP dissociation from Rho in vitro

While human D4-GDI has been previously shown to inhibit GDP dissociation from Rho family GTPases, we used purified recombinant proteins of wt and mt D4-GDI (V68L or V69A or both V68L and V69A) to directly compare their activities. Sf9 cell–expressed isoprenylated Rho were preloaded with [^{3}H]GDP, and the ability of wt D4-GDI, mt D4-GDIs, and wt plus mt D4-GDIs to inhibit dissociation of the nucleotide was determined. The dissociation of [^{3}H]GDP from Rho was totally blocked by wt D4-GDI (Fig. 10). The dissociation activity of [^{3}H]GDP from Rho by mt D4-GDI with both mutations of V68L and V69A was significantly low. On the other hand, the dissociation activity of mt D4-GDI with single mutation (V68L or V69A) was mild. Furthermore, inhibition of dissociation of [^{3}H]GDP

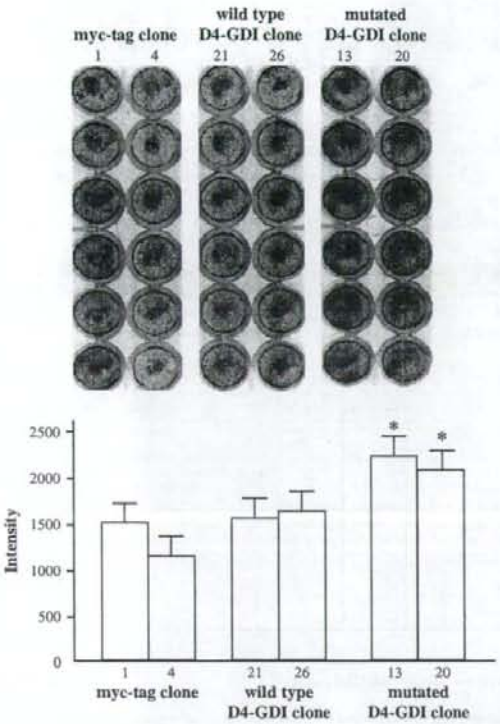

Figure 8. Augmentation of cell adhesion to human bone marrow stromal cells by mutated D4-GDI expression. The cell-adhesion activities of myc-tag clones, wild-type D4-GDI clones, and mutated D4-GDI clones were compared by using quantitative adhesion assays with fluorescent labeling of living cells (see Materials and Methods). In the upper panel, the unlabeled human stromal cells (invisible) attached to all wells and 2,7-bis-(2-carboxyethyl)-5 (and 6) carboxyfluorescein–labeled Raji cell clones, which adhered to the stromal cells were visible like black granules. The vertical line (six wells) represents data derived from one clone. In contrast to myc-tag clones 1 and 4, the mutated D4-GDI clones showed increased adhesiveness to human bone marrow stromal cells ($p < 0.05$). There was no significant difference in adhesion to human stromal cells between the myc-tag clones and wild-type D4-GDI clones. Data are represented as mean values. Error bars show standard error of mean. *Indicates a statistically significant increase ($p < 0.05$) as compared to data from myc-tag clone 1 or 4.

from Rho by wt D4-GDI was impaired by the addition of mt D4-GDI with both mutations of V68L and V69A. The negative effect by the addition of mt D4-GDI with single mutation of V68L or V69A to wt D4-GDI was not observed. As a result, the mt D4-GDI proteins with two mutations may function in a dominant negative manner in vitro.

Discussion

We identified two point mutations of D4-GDI in the human B-cell leukemic cell line. The region of D4-GDI containing these point mutations, which result in amino acid substitutions, is partially conserved in Rho-GDI family genes (Fig. 1). X-ray analysis of the three-dimensional structure of Rho-GDI suggested that these mutations of D4-GDI are in a β-sheet structure [36]. This region is at the back of the continuous surface adjacent to the isoprene-binding site of Rho-GDI, and could easily contact the bound GTPase and impart GDI activity. Robson protein secondary structure prediction suggested that these mutations may influence the β-sheet region [37]. The dissociation activity of GDP of D4-GDI with both mutations of V68L and V69A was decreased greater than the dissociation activity of mt D4-GDI with single mutation (V68L or V69A). Furthermore, dissociation of GDP from Rho by wt D4-GDI was significantly impaired by the addition of mt D4-GDI with both mutations of V68L and V69A. This result suggests that the D4-GDI proteins with two mutations may function in a dominant negative manner.

Thus, we speculated that this mt D4-GDI plays a role in development of hematological malignancy, and analyzed functions of mt D4-GDI in human leukemic cell invasion in vivo using a transplantation model of human leukemic cells into SCID mice. The SCID mice inoculated with Reh cells developed paraplegia 21 days after inoculation and all had died by days 26 to 27. The Reh cells infiltrated into bone marrow and around the spinal cord, with no involvement into peripheral blood, the spleen, liver, thymus or lymph nodes [1]. We identified mutations in the D4-GDI gene from human leukemic cells and showed that overexpression of mt D4-GDI in Raji cells accelerates leukemic cell invasion. Furthermore, we showed that overexpression of wt D4-GDI in Raji cells suppresses invasiveness. Additionally, there were no differences in cell growth rates among these clones, despite the altered invasiveness. On the other hand, cellular motile activity in the mt D4-GDI clones was augmented as compared with the myc-tag clones, and the motile activity of wt D4-GDI clones was significantly decreased. In the cell-adhesion assay, the mt D4-GDI clones showed increased adhesiveness to human bone marrow stromal cells. These findings indicate that the mt D4-GDI functions as a dominant negative molecule against endogenous D4-GDI.

Direct involvement of the Rho family in oncogenesis was discussed in a report [24]. Some GDS with a dbl-homology domain responsible for stimulating nucleotide exchange activity have been reported as potent oncogenes capable of transforming NIH-3T3 cells into a malignant phenotype (e.g., Dbl, Vav, and Lbc) [7,22]. Tiam1 was identified as an invasion-related gene and promoted leukemia progression through activation of the Rac signaling pathway [24]. In contrast to the function of Tiam1 in leukemic cells, Tiam1 and Rac have an invasion-suppressor role in epithelial cells [38]. Rho may also be involved in the increased mobility seen in metastasis through its control of the assembly of focal adhesions [39]. A study suggested that Rho regulates cadherin-mediated adhesion in small

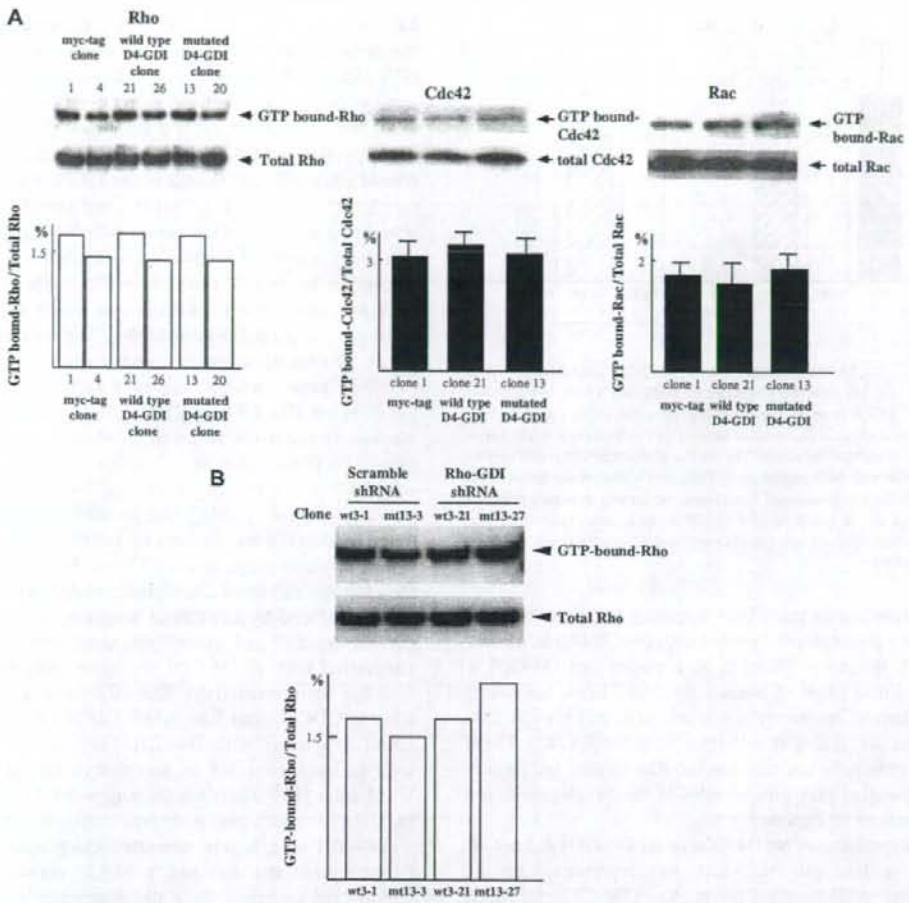

Figure 9. Detection of cellular guanine triphosphate (GTP)–bound Rho and Rac in leukemic cells. (**A**) Cells lysates were clarified by centrifugation, and equal volumes of lysates were incubated with 20 μg glutathione S-transferase (GST)–Rho-binding domain (RBD) beads. Beads were washed four times. Bound Rho proteins were detected by Western blotting using an anti-RhoA monoclonal antibody. The upper figures show the expressions of GTP-bound Rho and total Rho in all clones. The amounts of activated Rho (GTP-bound form) in all clones were almost the same. In order to quantitate the amount of GTP-bound Rho, a densitometric analysis was performed using NIH image version 1.62. The amount of RBD-bound Rho was normalized to the total amount of Rho in cell lysates for comparison of Rho activities (level of GTP-bound Rho) among different samples. The ratios of GTP-bound Rho to total Rho are shown in the lower graph. The GTP-bound Rho accounted for 1.3% to 1.6 % of total Rho in these six clones. There are no significant differences among the clones. The measurement of Rac activity was performed using the Rac Activation Assay kit (Cytoskeleton, Denver, CO, USA) according to the manufacturer's protocol. Amounts of activated Rac (GTP-bound form) in three clones were almost similar. In order to quantitate the amount of GTP-bound forms, a densitometric analysis was performed. The amount of GTP-bound Rac was normalized to the total amount of Rac in cell lysates for comparison of Rac activities among different samples. The ratios of GTP-bound Rac to total Rac are shown in the lower graph. The GTP- bound Rac accounted for 1.7% to 1.9% of total Rac in three clones, respectively. There are no significant differences among the clones. (**B**) Alterations of cellular GTP-bound Rho under the condition of decreased expression of Rho-GDI in leukemic cells with wt D4-GDI or mt D4-GDI overexpression. The pull-down assay for Rho using the wild-type or mutated D4-GDI clones (wt3-1, wt3-21, mt13-3, or mt3-27) with a vector of scramble shRNA or a vector for shRNA of Rho-GDI were done. As a result, the GTP-bound Rho in Rho-GDI knockdown mt D4 clones (mt13-27) was slightly increased compared with clones with scramble shRNA vector (wt3-1 and mt13-3) or Rho-GDI knockdown wt D4 clones (wt3-21).

cell lung carcinoma cells [40]. We reported that Rho activation augmented leukemic cell invasion through acceleration of cell adhesion, but not cell proliferation [1]. Itoh et al. [41] indicated rho-associated kinase played an essential part in tumor cell invasion, and that rho-associated kinase inhibitor may have potential as a therapy for prevention of malignant invasion and metastasis. In addition, Rho-GDI may also play a role in cancer invasion and metastasis

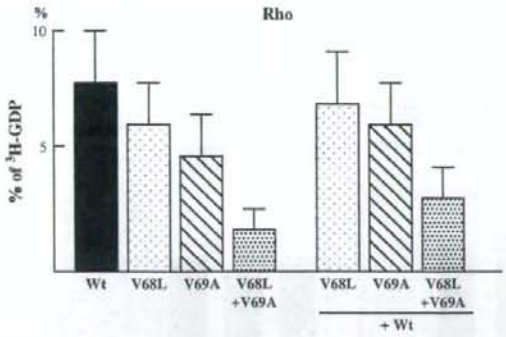

Figure 10. [³H]GDP dissociation assay of Rho. The inhibitory activities of mutated (mt) D4 with two mutations of V68L and V69A on the dissociation of [³H]GDP from isoprenylated Rho were less active than wild-type (wt) D4. The decreased dissociation activity of D4 with each single mutation (V68L orV69A) was slight. The results of the inhibitory activities of wt plus mt D4 with both mutations of V68L and V69A on the dissociation of [³H]GDP from isoprenylated Rho showed mt D4 was dominant negative of wt D4 on the dissociation of [³H]GDP from isoprenylated Rho. The dominant negative effect was not observed in the D4 with single mutation of V68L orV69A.

via involvement in the CD44 signaling pathway, because Rho-GDI coimmunoprecipitated with the CD44-ERM complex [42]. Recently, Zhang et al. reported that D4-GDI is expressed in a panel of breast cancer cell lines, but not in benign-derived mammary epithelial cells, and the D4-GDI modulates breast cancer cell–invasive activities [43]. These findings obviously indicate that the Rho family and its regulatory proteins play critical roles in the development and progression of malignancy.

Overexpression of wt D4-GDI or mt D4-GDI did not alter Rho or Rac activity, which was represented by the amount of GTP-bound Rho or Rac. D4-GDI functions both to inactivate Rho, via inhibition of the GDP dissociation from Rho, and to activate Rho, via suppression of the GTPase activity of Rho itself. Furthermore, D4-GDI has weaker GDP dissociation inhibitory activity (10-fold less) than Rho-GDI. Recently, Zhang et al. [43] reported that the activation status of Rac1, Cdc42, and RhoA was not altered as a result of D4-GDI depletion. In addition, 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]. Although recombinant D4-GDI binds to purified Rac1, Cdc42, and RhoA, there is no evidence showing that they can form stable complexes in vivo [43]. Thus, the lack of changes in Rho or Rac activity in response to exogenous expression of wt D4-GDI or mt D4-GDI may be explained. On the other hand, the GTP-bound Rho in mt D4-GDI clone with Rho-GDI was slightly increased in contrast to mt D4-GDI clone without Rho-GDI knockdown or wt D4-GDI clones with/without Rho-GDI knockdown. These results suggest that the altered phenotypes of leukemic cells may be partially caused by Rho activation due to mt D4-GDI expression. However, the invasiveness, motility, and adhesion activity of mt D4-GDI clone with Rho-GDI knockdown was not changed in spite of the mild Rho activation in the mt D4 clone. Ishizaki et al. [44] recently report that combined disruption of both the Rho-GDI and D4-GDI genes in mice resulted in reduction of marginal zone B cells in the spleen, retention of mature T cells in the thymic medulla, and a marked increase in eosinophil numbers. Our results may be explained by the fact that the level of Rho-GDI knockdown was insufficient in contrast to the null mutation of Rho-GDI gene.

It was shown that D4-GDI is specifically cleaved at two positions (residues 18–19 and 54–55) by two different apoptosis proteases, caspase-3 and caspase-1, respectively [17,45]. These consensus cleavage sequences are not present in either Rho-GDI or Rho-GDIγ. A truncated D4-GDI cleaved by caspase-1 is unable to effectively bind and regulate Rho family members. D4-GDI is a target protein of caspase-3 in the process of anti–IgM-mediated or Fas-dependent apoptosis [17,46]. The positions of point mutations found in D4-GDI are residues 68 and 69. Therefore, the positions of these mutations are 13 and 49 amino acids from the cleavage sequence. No significant differences in the apoptosis induced by anti-cancer reagents, i.e., methotrexate, cyclohexamide, and vincristine, were seen in Raji cells transferred with mt D4-GDI transgene (data not shown).

It has been reported that Rho-GDI forms a complex with Rho A, CDC42, and Rac, while CDC42 and Rac was not found to interact with D4-GDI. Furthermore, stimulation with phorbol ester led to phosphorylation of D4-GDI in U937 cells [15]. Their results suggested that D4-GDI can regulate specific signal pathways in hematopoietic cells.

D4-GDI is a highly abundant cytoplasmic protein in lymphocytes, and has had a highly conserved primary amino acid sequence since the divergence of mammalian species. However, D4-GDI–deficient mice and in vitro embryonal stem cell differentiation analysis indicated D4-GDI expression is not essential for hematopoiesis and did not clarify its function in hematopoietic cells [47,48]. Our results indicate that D4-GDI overexpression in transformed cells changes cell motility, cell adhesion, and invasiveness in some organs. In normal lymphocytes, D4-GDI may have a subtle, yet crucial, function related to cell motility and adhesion.

Li et al. [49] reported that D4-GDI might be involved in the progression of human cutaneous T-cell lymphoma using a cDNA microarray in the clonally related T-cell lines derived from different stages of a progressive T-cell lymphoma involving skin. They found the D4-GDI gene to be one of the downregulated genes in cells from an advanced, clinically aggressive stage lymphoma, in contrast to cells from an earlier, clinically indolent stage of lymphoma. Expression of D4-GDI mRNA in cells derived from the aggressive stage lymphoma was shown to be markedly decreased as compared with cells derived from

the earlier-stage lymphoma. This result is compatible with our data, showing overexpression of wt D4-GDI to reduce the invasiveness of human leukemic cells. Thus, D4-GDI may assure the progression and invasion of human leukemia through its mutations and/or its downregulation.

Accumulating recent evidence shows that D4-GDI is expressed not only in hematopoietic tissues, but also in non-hematopoietic neoplasms. Results of cDNA microarray analyses revealed that D4-GDI is upregulated in ovarian [50], and downregulated in bladder, carcinomas [51]. On the other hand, Theodorescu et al. [52] found that D4-GDI protein expression in bladder tumors is reduced as a function of bladder tumor progression. This result has suggested that D4-GDI is a metastasis suppressor gene in models of bladder cancer. In contrast, the results of Zhang et al. [43] show that increased expression of D4-GDI promotes cell invasiveness in breast cancer cells. These results suggest that the D4-GDI may have certain roles in the progression of different types of cancer. Thus, it is reasonable to propose that D4-GDI may be involved more generally in the invasive phenotype of human cancer.

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ORIGINAL ARTICLE: CLINICAL

Outcome of childhood B-cell non-Hodgkin lymphoma and B-cell acute lymphoblastic leukemia treated with the Tokyo Children's Cancer Study Group NHL B9604 protocol

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Abstract

From June 1996 to January 2001, 91 patients with B-cell non-Hodgkin lymphoma or B-cell acute lymphoblastic leukemia up to 18 years of age were enrolled in Tokyo Children's Cancer Study Group (TCCSG) NHL B9604 protocol study. Five-day intensive chemotherapy courses including high-dose methotrexate and high-dose cyclophosphamide were used for localized disease (Groups A and B). High-dose cytarabine was added for advanced disease (Groups C and D). Fifteen patients experienced an adverse event. There were three induction failures, eight relapses (three local, four bone marrow (BM), one BM + local), two toxic deaths and two second malignant neoplasm. Event-free survival at 6 years in Group D and in all patients was 82.4% ± 9.2% and 81.9% ± 4.4%, respectively. The TCCSG NHL B9604 protocol achieved an excellent treatment outcome especially in patients with the most advanced disease (Group D: high BM blast cell burden and/or central nervous system involvement).

Keywords: *B-NHL, B-ALL, intensified chemotherapy, TCCSG NHL B9604*

Introduction

The malignant cells of B-cell (surface immunoglobulin-positive [Ig+]) non-Hodgkin lymphoma (B-NHL) and B-cell acute lymphoblastic leukemia (B-ALL), which are classified as Burkitt lymphoma/leukemia in WHO classification [1], share morphologic, immunophenotypic, cytogenetic and clinical features and are considered to represent a continuum of the same disease. Diffuse large B-cell lymphoma is a distinct disease entity from Burkitt lymphoma/leukemia. However, the treatment for patients with large cell lymphoma is the same as that for patients with Burkitt histology. In early trials, children with advanced B-NHL and B-ALL had a worse outcome characterized by early recurrences despite a high initial complete remission rate [2,3]. After the introduction of short, intensive therapy courses primarily based on cyclophosphamide (CY), methotrexate (MTX), and intrathecal (i.t.) therapy, the prognosis of these mature B-cell neoplasms has improved significantly [4–11].

Most previous clinical experiences about childhood B-NHL and B-ALL were reported from European and North American study groups, however, there are few data on Japanese or Asian patients. We report here the treatment and results of 91 Japanese children with B-NHL or B-ALL registered to the Tokyo Children's Cancer Study Group (TCCSG) NHL B9604. The aim of this study

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was to evaluate the efficacy and safety of the short and intensive chemotherapy regimen designed by TCCSG for childhood B-NHL or B-ALL.

Patients and methods

Children and adolescents up to 18 years of age newly diagnosed with B-NHL or B-ALL were eligible for this study. From June 1996 to January 2001, 91 patients with B-NHL or B-ALL were enrolled in this study. Diagnosis was made by pathologists at a regional hospital and registration was made based on the regional diagnosis. A central review was performed retrospectively by the reference laboratory according to the WHO classification [1]. The St. Jude system was used for disease staging [12]. Patients were stratified into four groups according to stage, resection status, bone marrow (BM) blast cell burden and central nervous system (CNS) involvement as shown in Figure 1.

Five-day intensive chemotherapy courses including high-dose MTX (HD-MTX) and high-dose CY (HD-CY) were used for Groups A and B. High-dose cytarabine (HD-AraC) was added for Groups C and D. Patients in Groups B, C and D received a 5-day cytoreductive prephase before the first course was administered (Table I). Courses were administered at 3–4-week intervals. The number of treatment courses was three in Group A, six in B, six in C and seven in D, respectively. Treatment duration ranged from 10 to 27 weeks (Figure 1).

Events were defined as induction failure, relapse, death in induction, death in remission and second malignant neoplasm. Survival curves were calculated by the Kaplan–Meier method. Toxicities were graded according to National Cancer Institute Common Toxicity Criteria version 2. All treatments were performed with informed consent from the patients' guardians.

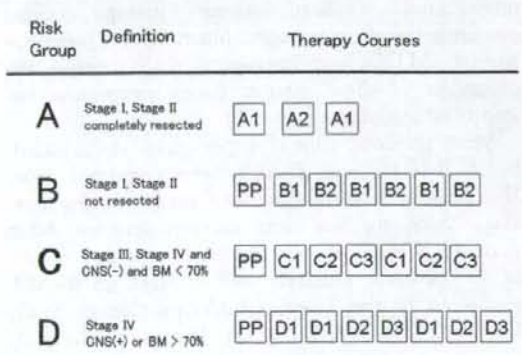

Figure 1. Treatment strategy. Patients were stratified into four risk groups; A, B, C and D. The composition of therapy courses is shown in Table I.

Results

The clinical characteristics of the 91 patients are shown in Table II. The percentage in Group A patients in our series was quite small (3.3%) compared with that of equal disease status patients in other studies (7–17%) [6,8,11]. A retrospective central review was performed in 64.8% (59/91) of the enrolled patients and the concordance rate was 88.1% (52/59). Fifteen patients experienced an adverse event. There were three induction failures, eight relapses (three local, four BM, one BM + local), two toxic deaths and two second malignant neoplasm. Nine patients died of induction failure in three, relapse in four and toxicity in two patients [intracranial hemorrhage (ICH) and sepsis]. Two patients developed a second malignant neoplasm. Grade 4 non-hematological toxicity was noted in 4 cases. Event-free survival (EFS) and overall survival (OS) were calculated as follows: EFS and OS in total were $81.9\% \pm 4.4\%$ and $90.1\% \pm 3.1\%$, respectively. EFS was $66.7\% \pm 27.2\%$ in Group A ($n=3$), $95.8\% \pm 4.1\%$ in Group B ($n=25$), $77.6\% \pm 6.3\%$ in Group C ($n=46$) and $82.4\% \pm 9.2\%$ in Group D ($n=17$) (Figure 2). OS was in 100% in Groups A and B ($n=28$), $86.8\% \pm 5.0\%$ in Group C and $82.4\% \pm 9.2\%$ in Group D.

Discussion

In this article, we reported the outcome of 91 childhood B-NHL and B-ALL patients treated with TCCSG NHL B9604 protocol. OS and EFS at 6 years in total were $90.1\% \pm 3.1\%$ and $81.9\% \pm 4.4\%$, respectively. OS of our study was comparable with SFOP study [8], BFM study [6] and UKCCSG study [7] and better than CCG study [9] or Venezuela study [11] whereas the observation period of our study was longer than other studies. Although the EFS of our study was worse than the SFOP study [8] and BFM study [6], it was comparable with the UKCCSG study [7] and better than the CCG study [9], POG study [4] and Venezuela study [11]. Especially in patients with the most advanced disease (Group D: high BM blast cell burden and/or CNS involvement), EFS was $82.4\% \pm 9.2\%$. This was comparable with the SFOP study [8] and BFM study [6]. The overall good prognosis of this study is thought to be mainly due to the relatively larger dose of chemotherapeutic drugs and intensified regimens resulting in a good outcome.

There were 15 event cases in this study, as shown in Table III. Among these cases, the diagnosis of two relapsed patients (UPN 132,146), whose diagnosis was DLBCL by the regional hospital, was corrected to B-LBL by retrospective central review. These

Table I. Therapeutic regimen of TCCSG NHL B9604 protocol.

Group A	
Block A1:	
PDN 60 mg/m^2 P.O.	Days 1–5
MTX 3 g /m^2 6 h div with CFR	Days 1
CY 250 mg/m^2 1 h div	Days 2–5
VP16 100 mg/m^2 2 h div	Days 2–5
MH i.t.	Days 1
Block A2:	
PDN 60 mg/m^2 P.O.	Day 1–5
VCR 1.5 mg/m^2	Day 1
AraC150 mg/m^2 × 6 1 h div q12 h	Day 1–3
CY 1 g/m^2 2 h div	Day 2,3
MH i.t.	Day 1
Group B	
Block P(Prephase):	
PDN 60 mg/m^2 P.O.	Day 1–5
CY 200 mg/m^2 1 h div	Day 1, 2
MH i.t.	Day 1
Block B1:	
PDN 60 mg/m^2 P.O.	Day 1–5
MTX 3 g/m^2 6 h div with CFR	Day 1
CY 250 mg/m^2 1 h div	Day 2–5
VP16 100 mg/m^2 2 h div	Day 2–5
Epi 50 mg/m^2 1 h div	Day 5
MH i.t.	Day 1
Block B2:	
PDN 60 mg/m^2 P.O.	Day 1–5
VCR 1.5 mg/m^2	Day 1
AraC 150 mg/m^2 × 6 1 h div q12 h	Day 1–3
CY 1 g/m^2 2 h div	Day 2,3
Epi 50 mg/m^2 1 h div	Day 5
MH i.t.	Day 1
Group C	
Block P:	
PDN 60 mg/m^2 P.O.	Day 1–7
VP16 100 mg/m^2 1 h div	Day 5–7
MHC i.t.	Day 1,6
Block C1:	
Dex 10 mg/m^2 P.O.	Day 1–5
MTX 3 g/m^2 24 h div with CFR	Day 1
CY 250 mg/m^2 × 6 1 h div q12 h	Day 2–4
VP16 100 mg/m^2 2 h div	Day 2–5
(only day 2,3 in the first course)	
Epi 60 mg/m^2 1 h div	Day 5
MHC i.t.	Day 5 (in the first course)
MH i.t.	Day 1(in the second course)
Block C2:	
Dex 10 mg/m^2 P.O.	Days 1–5
VCR 1.5 mg/m^2	Days 1
AraC 150 mg/m^2 × 6 1 h div q12 h	Days 1–3
CY 1 g/m^2 2 h div	Days 2–4
Epi 60 mg/m^2 1 h div	Days 5
MHC i.t.	Day 1
MH i.t.	Day 8 (only in the first course)
Block C3:	
Dex 10 mg/m^2 P.O.	Day 1–5
VDS 3 mg/m^2	Day 1
VP16 100 mg/m^2 2 h div	Day 2–5
AraC 2 g/m^2 3 h div q12 h	Day 1–3
Epi 60 mg/m^2 1 h div	Day 5
MH i.t.	Day 1
MH i.t.	Day 8 (only in the first course)
Group D:	
Block P: the same as in Group C	
Block D1:	
Dex 10 mg/m^2 P.O.	Day 1–5
MTX 3 g/m^2 24 h div with CFR	Day 1
CY 300 mg/m^2 × 6 1 h div q12 h	Day 2–4
(only day 2,3 in the first course)	
Epi 80 mg/m^2 1 h div	Day 5
MHC i.t.	Day 5 (in the first course)
MH i.t.	Day 1 (in the second course)
MHC i.t.	Day 8 (in the second course)
MH i.t.	Day 1,8 (in the third course)
Block D2:	
Dex 10 mg/m^2 P.O.	Day 1–5
VCR 1.5 mg/m^2	Day 1
AraC 150 mg/m^2 × 6 1 h div q12 h	Day 1–3
CY 1 g/m^2 2 h div	Day 2–4
Epi 80 mg/m^2 1 h div	Day 5
MHC i.t.	Day 1 (in the first course)
MH i.t.	Day 8 (in the first course)
MH i.t.	Day 1,8 (in the second course)
Block D3:	
Dex 10 mg/m^2 P.O.	Day 1–5
VDS 3 mg/m^2	Day 1
VP16 100 mg/m^2 2 h div	Day 2–5
AraC 2 g/m^2 3 h div q12 h	Day 1–3
Epi 80 mg/m^2 1 h div	Day 5
MH i.t.	Day 1,8

Cranial irradiation for patients with CNS involvement at onset
24 Gy for older than 2 years old
18 Gy for 1 year old
0 Gy for less than 1 year old

Cumulative drug dosage in each group

Group	A	B	C	D
CY (g/m^2)	4	9.4	9	11.4
VP16 (mg/m^2)	800	1200	1700	2100
MTX (g/m^2)	6	9	6	9
CA (g/m^2)	0.9	2.7	25.8	28.8
Epi (mg/m^2)	0	300	360	560

PDN, prednisolone; CFR, citrovorum factor rescue; VP16, etoposide; MH, MTX and hydrocortisone; VCR, vincristine; AraC, cytarabine; q12h, every 12 h; Epi, epirubicine; MHC, MH and AraC; Dex, dexamethazone; VDS, vindesine.

Table II. Clinical characteristics of 91 patients treated with TCCSG NHL B9604 protocol.

Registration period	April 1996–January 2001
Observation period	0–103 months (median 60 months)
Sex (Male/Female)	64/27
Age range	0.9–16.8 years (median 9.4 years)
Pathological diagnosis	Burkitt, 45
	Large cell, 26
	B-ALL, 9
	B-NHL not further specified, 6
	Others, 5
Primary site	Head and neck: 32; chest: 3
	Abdomen: 36; bone: 8
	B-ALL: 9; others: 3
Disease stage	I: 5; II: 23; III: 25; VI: 38
Risk group	A: 3; B: 25; C: 46; D:17

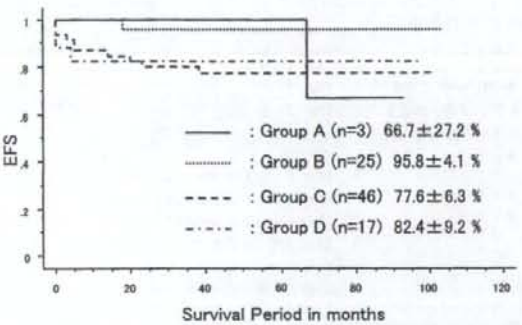

Figure 2. EFS according to the risk groups. EFS was 66.7% ± 27.2% in Group A ($n=3$), 95.8% ± 4.1% in Group B ($n=25$), 77.6% ± 6.3% in Group C ($n=46$) and 82.4% ± 9.2% in Group D ($n=17$).

patients were rescued with further chemotherapy for B-LBL. These relapses were considered due to inadequate treatment duration rather than drug resistance. In Group A, one patient (UPN 100) whose primary site was the tonsils developed diffuse large B-cell lymphoma at the cecum 5 years after initial diagnosis. Although we could not determine whether this lesion was relapse or secondary neoplasm, the EFS of Group A patients became low because of this rare event. Such a late relapse as in this case was also reported in the SFOP study [8]. Aside from these three cases, the 12 event cases consisted of three induction failures, five relapses, two toxic deaths and two second malignant neoplasm. All three induction failures and four BM involved relapsed patients died of disease progression in spite of further chemotherapy or stem cell transplantation. These cases may have required a quite different treatment concept for the first-line therapy based on stratification with tumor biology profiling. One patient (UPN 44) showed massive ICH at initial presentation and died 2 days after

Table III. Event cases in TCCSG NHL B9604 protocol.

UPN	Age	Sex	Stage	Group	Histology (central review)	Event	Remission period	Outcome	Cause of death	Survival period
11	15	F	III	C	DLBCL (DLBCL)	Secondary malignancy	14	Alive	N.A.	88+
22	5	F	III	C	Burkitt (Burkitt)	Induction failure	0	Dead	Disease progression	9
39	14	M	III	C	Burkitt (Burkitt)	Relapse (BM)	3	Dead	Disease progression	6
44	9	M	IV	D	ALL	ICH	0	Dead	ICH	0
57	11	M	IV	D	Burkitt (Burkitt)	Relapse (BM)	4	Dead	Disease progression	6
58	3	F	IV	C	Burkitt (Burkitt)	Induction failure	0	Dead	Disease progression	6
74	15	M	III	C	Burkitt (Burkitt)	Relapse (BM + local)	6	Dead	Disease progression	7
100	13	F	I	A	DLBCL (DLBCL)	Relapse (cecum)	67	Alive	N.A.	71+
102	10	F	IV	C	ALL	Relapse (BM)	6	Dead	Disease progression	11
103	2	M	III	C	Burkitt (Burkitt)	Sepsis	0	Dead	Sepsis	0
132	7	M	II	B	DLBCL (B-LBL)	Relapse (BM)	33	Alive	N.A.	66+
141	14	M	III	C	Medium (N.D.)	Relapse (local)	20	Alive	N.A.	77+
146	7	F	IV	C	DLBCL (B-LBL)	Relapse (local)	23	Alive	N.A.	60+
149	15	M	III	C	MLBCL (MLBCL)	Secondary malignancy	38	Alive	N.A.	62+
154	8	M	IV	D	Burkitt (Burkitt)	Induction failure	0	Dead	Disease progression	10

DLBCL, diffuse large B cell lymphoma; MLBCL, mediastinal large B-cell lymphoma; ICH, intracranial hemorrhage; N.A., not applicable; N.D., not done.
Age is shown in years. Remission period and survival period are shown in months. A plus sign indicates that the patient is still alive.

admission. We do not think this was therapy-related toxic death. Toxic death was reported in another patient (UPN 103) who died of sepsis during remission induction therapy. Although Grade 4 non-hematological toxicity was noted in four cases in addition to this patient, this intensified treatment regimen was well tolerated. Two second malignant neoplasm (myelodysplastic syndrome and acute myeloid leukemia) were observed 14 and 38 months after diagnosis. These patients survived with BM transplantation from unrelated donors. As we used a relatively larger dose of chemotherapeutic drugs in this study, we have to cautiously observe the development of a second malignant neoplasm among patients treated with this protocol.

In recent childhood lymphoma studies, international collaborations are essential because large scale studies are needed to prove improved outcome compared with current good one. To participate in these international studies, it is important for us (Japanese or Asian people) to confirm short, intensive chemotherapy for B-NHL and B-ALL is safe and effective regardless of racial differences. We elucidated such short, intensive chemotherapy for B-NHL and B-ALL was safe and effective for Japanese children. Treatment reduction is a main theme of childhood B-NHL therapy. Recently, Patte et al. [13] reported results of FAB/LMB96 trial for intermediate risk B-NHL patients. In this report, they elucidated a four-course treatment is enough for these patients with initial good response. Several studies for B-NHL have attempted therapy reduction to decrease toxicity, however, the dose reduction is associated with an inferior outcome for advanced stage disease so far [14,15].

The TCCSG NHL B9604 protocol achieved an excellent treatment outcome, especially in patients with the most advanced disease (Group D: high BM blast cell burden and/or CNS involvement). Although we have to cautiously observe the development of late adverse effects in treated patients, several study attempts have not succeeded in appropriate therapy reduction without jeopardizing survival and intensified regimen is, at least tentatively, needed for a good prognosis for advanced stage patients.

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Inducible Expression of Chimeric EWS/ETS Proteins Confers Ewing's Family Tumor-Like Phenotypes to Human Mesenchymal Progenitor Cells▽†

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Ewing's family tumor (EFT) is a rare pediatric tumor of unclear origin that occurs in bone and soft tissue. Specific chromosomal translocations found in EFT cause EWS to fuse to a subset of ets transcription factor genes (ETS), generating chimeric EWS/ETS proteins. These proteins are believed to play a crucial role in the onset and progression of EFT. However, the mechanisms responsible for the EWS/ETS-mediated onset remain unclear. Here we report the establishment of a tetracycline-controlled EWS/ETS-inducible system in human bone marrow-derived mesenchymal progenitor cells (MPCs). Ectopic expression of both EWS/FLI1 and EWS/ERG proteins resulted in a dramatic change of morphology, i.e., from a mesenchymal spindle shape to a small round-to-polygonal cell, one of the characteristics of EFT. EWS/ETS also induced immunophenotypic changes in MPCs, including the disappearance of the mesenchyme-positive markers CD10 and CD13 and the up-regulation of the EFT-positive markers CD54, CD99, CD117, and CD271. Furthermore, a prominent shift from the gene expression profile of MPCs to that of EFT was observed in the presence of EWS/ETS. Together with the observation that EWS/ETS enhances the ability of cells to invade Matrigel, these results suggest that EWS/ETS proteins contribute to alterations of cellular features and confer an EFT-like phenotype to human MPCs.

Ewing's family tumor (EFT) is a rare childhood cancer arising mainly in bone and soft tissue. Since EFT has a poor prognosis, it is important to elucidate the underlying pathogenic mechanisms for establishing a more effective therapeutic strategy. EFT is characterized by the presence of chimeric genes composed of EWS and ets transcription factor genes (ETS) formed by specific chromosomal translocations, i.e., EWS/FLI1, t(11;22)(q24;q12); EWS/ERG, t(21;22)(q12;q12); EWS/ETV1, t(7;22)(p22;q12); EWS/E1AF, t(17;22)(q12;q12); and EWS/FEV, t(2;22)(q33;q12) (26). The products of these chimeric genes behave as aberrant transcriptional regulators and are believed to play a crucial role in the onset and progression of EFT (3, 36). Indeed, recent studies have revealed that the induction of EWS/FLI1 proteins can trigger transformation in certain cell types, including NIH 3T3 cells (36), C2C12 myoblasts (12), and murine primary bone marrow-derived mesenchymal progenitor cells (MPCs) (6, 45, 52). However, studies have also indicated that overexpression of EWS/FLI1 provokes apoptosis and growth arrest in mouse normal embryonic fibroblasts and primary human fibroblasts (10, 31), hence hampering understanding of the precise role of EWS/ETS proteins in the development of EFT. The function of EWS/ETS proteins would be greatly influenced by cell type, and thus the cells that can originate EFTs might be more susceptible to the tumorigenic effects of EWS/ETS.

Although the cell origin of EFT is still unknown, the expression of neuronal markers in spite of the occurrence in bone and soft tissues has kept open the debate as to a potential mesenchymal or neuroectodermal origin. As described above, ectopic expression of EWS/FLI1 results in dramatic changes in morphology and the formation of EFT-like tumors in murine primary bone marrow-derived MPCs but not in murine embryonic stem cells (6, 45, 52), supporting the notion that MPCs are a plausible cell origin of EFT (45). However, others argue that MPCs cannot be considered progenitors of EFT without further evidence of similarity between human EFT and MPC-EWS/FLI1-induced tumors in mice (29, 46).

The development of experimental systems using murine species is useful for elucidating the mechanisms behind the pathogenesis of EFT. However, several differences between human and murine systems cannot be ignored; these differences include the expression patterns of surface antigens in MPCs, for instance (7, 44, 51, 53). Moreover, human cells are difficult to transform in vitro, and the transformed cells of mice seem to produce a more aggressive tumor than those of hu-

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TABLE 1. Cell lines used in this study and fusion transcript types

Cell line	Diagnosis	Fusion transcript type	Reference
EES-1	EFT	EWS/FLI1 type I	20
SCCH196	EFT	EWS/FLI1 type I	21
RD-ES	EFT	EWS/FLI1 type II	5
SK-ES1	EFT	EWS/FLI1 type II	5
NCR-EW2	EFT	EWS/FLI1 type II	19
NCR-EW3	EFT	EWS/E1AF	19
W-ES	EFT	EWS/ERG	13
NB69	NB		15
NB9	NB		15
GOTO	NB		47
NRS-1	RMS	PAX3/FKHR	40

mans (1). The findings suggest the existence of undefined cell-autonomous mechanisms that render human cells resistant to malignant transformation. Therefore, the use of human cell models is ideal for clarifying how EFT develops. Models of the onset of EFT have been generated using primary fibroblasts (31) and rhabdomyosarcoma cells (23). However, these cell types are not appropriate for studying the origins of EFT, and a model that precisely recapitulates EWS/ETS-mediated EFT formation is required.

UET-13 cells are obtained by prolonging the life span of human bone marrow stromal cells by use of the retroviral transgenes hTERT and E7 (38, 50), retain the ability to differentiate into not only mesodermal derivatives but also neuronal progenitor-like cells, and are considered a good model for studying the cellular events in human MPCs. Therefore, we have examined the biological effect of EWS/ETS in human MPCs by use of UET-13 cells by exploiting tetracycline-inducible systems for expressing EWS/ETS (EWS/FLI1 and EWS/ERG). Here we report that overexpression of EWS/ETS mediates an EFT-like phenotype, including morphology, immunophenotype, and gene expression profile, with enhancement of the Matrigel invasion ability of UET-13 cells.

MATERIALS AND METHODS

Cell cultures and establishment of UET-13TR-EWS/ETS cell lines. UET-13 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% Tet system approved fetal bovine serum (T-FBS) (Takara) at 37°C under a humidified 5% CO_2 atmosphere. EFT cell lines (EES-1 [20], SCCH196 [21], RD-ES and SK-ES1 [5], NCR-EW2 and NCR-EW3 [19], and W-ES [13]) and neuroblastoma (NB) cell lines (NB69 and NB9 [15] and GOTO [47]) were cultured in RPMI 1640 with 10% FBS. A rhabdomyosarcoma cell line, NRS-1 (40), was cultured in Eagle's minimal essential medium with 10% FBS. The cell lines used in this study are listed in Table 1.

UET-13 cells were seeded at a density of 5×10^4 cells per well in 24-well tissue culture plates 1 day prior to transfection. For introducing the tetracycline-inducible system, UET-13 cells were transfected with pcDNA6-TR (Invitrogen) by use of Lipofectamine 2000 (Invitrogen). After 72 h, the medium was replaced with fresh medium containing 200 μg/ml of blasticidin S (Invitrogen). Individual resistant clones were selected for a month and designated UET-13TR cells. UET-13TR cells were further transfected with pcDNA4-EWS/ETSs constructed as described below, and individual resistant clones were selected in DMEM containing 10% T-FBS and 200 to 300 μg/ml of Zeocin (Invitrogen). The Zeocin-resistant clones were expanded and tested for the induction of EWS/ETS expression upon the addition of tetracycline by use of reverse transcription-PCR (RT-PCR) as described below.

Plasmid construction. A gateway cassette (bases 1 to 1705) was amplified from pBLOCK-iT3-DEST (Invitrogen) by PCR, and the PCR product was inserted into the EcoRV site of pcDNA4-TO (Invitrogen) (termed pcDNA4-DEST). Since the type II EWS/FLI1 is a stronger transactivator than the type I product (32), we used the type II variant in the present study. EWS/ERG was isolated from W-ES, an EFT cell line, joining EWS exon 7 and ERG exon 9. Full-length EWS/FLI1 type II and EWS/ERG cDNAs were amplified from cDNAs prepared from NCR-EW2 and W-ES cells, respectively, by PCR as described below and cloned into the XmnI-EcoRV sites of pENTR11 (Invitrogen). The resulting pENTR11-EWS/ETSs were recombined with pcDNA4-DEST by use of LR recombination reaction as instructed by the manufacturer (Invitrogen) to construct the tetracycline-inducible EWS/ETS expression vector pcDNA4-EWS/ETSs.

Western blot analysis. UET-13 transfectants were cultivated with or without 3 μg/ml of tetracycline for 72 h. Western blot analysis was performed as previously described (37). Briefly, the cell lysates were prepared and separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred onto a polyvinylidene difluoride membrane. The membranes were blocked with 5% skimmed milk in phosphate-buffered saline (PBS) containing 0.01% Tween 20 (Sigma) and incubated with primary antibodies. As the primary antibodies, anti-Fli-1, anti-Erg-1/2/3 (Santa Cruz Biotechnology), and anti-actin (Sigma) were used. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G (IgG) antibodies (DakoCytomation) were used as secondary antibodies. Blots were detected by chemiluminescence using an ECL Plus Western blotting detection system (GE Healthcare Bio-Science Corp.) and exposed to X-ray film (Kodak) for 5 to 30 min.

MTT assay and detection of apoptosis. Growth curves of UET-13 transfectants were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (18). The apoptosis was detected using an annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Biovision) according to the manufacturer's instructions and analyzed by flow cytometry (Cytomics FC500; Beckman Coulter).

Immunofluorescence analysis. After 1 week of culture in the absence or presence of tetracycline, UET-13 cells and the transfectants were harvested with 0.25% trypsin plus EDTA (IBL). The cells (2×10^5) were incubated with mouse monoclonal antibodies for 20 min. In the case of fluorescence-labeled antibodies, the cells were washed with PBS and then analyzed. In the case of primary unconjugated mouse antibodies, the cells were washed and then incubated with FITC-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories) for 20 min. Cell fluorescence was detected using a Cytomics FC500 instrument as described previously (27).

Antibodies against the following human antigens were used: CD10, CD13, CD14, CD29, CD34, CD40, CD44, CD45, CD49e, CD54, CD56, CD61, CD90, CD105, CD117, and CD166 from Beckman Coulter; CD73 from BD Biosciences-Pharmingen; CD55 from Abcam; CD59 from Cedarlane Laboratories; and CD133 and CD271 from Miltenyi Biotec GmbH.

Immunocytochemistry. Cells were grown on collagen type I-coated cover glasses (Iwaki). After 72 h with or without tetracycline, cells were fixed for 30 min in 4% paraformaldehyde and permeabilized in PBS containing 0.2% Triton X-100 (Sigma) for 30 min. Subsequently, they were washed with PBS and blocked in PBS containing 0.1% Triton X-100 and 1% bovine serum albumin (Sigma) for 30 min before being incubated with a monoclonal anti-CD99 antibody, i.e., 12E7 (1:100) (DakoCytomation) or O13 (1:200) (Thermo), and polyclonal anti-Fli-1 antibody (1:100) (Santa Cruz) for 1 h. Bound antibodies were visualized with appropriate secondary antibodies, i.e., Alexa Fluor 488 goat anti-mouse IgG (heavy plus light chains) highly cross-adsorbed and Alexa Fluor 546 goat anti-rabbit IgG (heavy plus light chains) highly cross-adsorbed (Invitrogen) for 1 h at 1:300. Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) or propidium iodide (PI) (Sigma). For the visualization of whole cells, cells were treated with Celltracker Blue (Invitrogen) for 30 min and then fixed. Fluorescence was observed and analyzed using a confocal laser scanning microscope and image software (either FV500 from Olympus or LSM510 from Carl Zeiss). Precise measurements of cell size, nuclear size, and the nucleus-to-cytoplasm (N/C) ratio were performed using Image J (16).

RT-PCR analysis. Total RNA was extracted from cells by use of an RNeasy kit (Qiagen) and reverse transcribed using a first-strand cDNA synthesis kit (GE Healthcare Bio-Science Corp). RT-PCR was performed with a HotstarTaq master mix kit (Qiagen). As an internal control, human GAPDH cDNA was also amplified. The sequences of gene-specific primers for RT-PCR were as follows: for EWS/FLI1 (forward), 5′-ATGGCGTCCACGGATTACAGTACCT-3′; for EWS/FLI1 (reverse), 5′-GGGTCTTCTTTGACACTCAATCG-3′; for EWS/ERG (forward), 5′-ATGGCGTCCACGGATTACAGTACCT-3′; for EWS/ERG (reverse), 5′-TTAGTAGTAAGTGCCCAGATGAGAA-3′; for GAPDH (forward), 5′-CCACCCATGGCAAATTCCATGGCA-3′; and for GAPDH (reverse), 5′-TCTAGACGGCAGGTCAGGTCCACC-3′. PCR products were electrophoresed with a 1% agarose gel and stained with ethidium bromide.

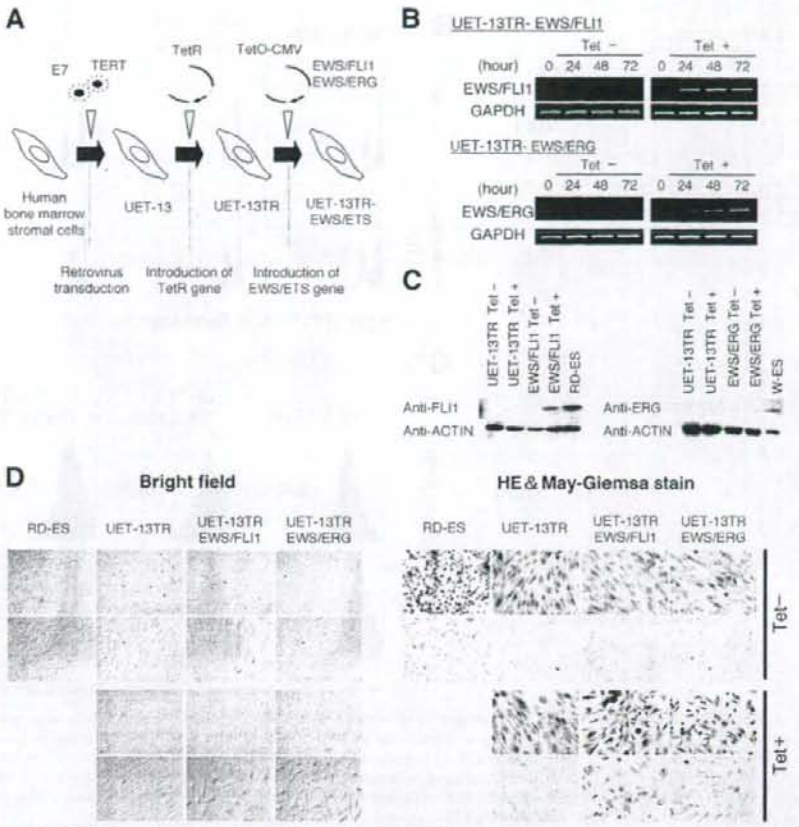

FIG. 1. The effect of EWS/ETS on the morphology of UET-13 cells. (A) The establishment of a tetracycline-inducible EWS/ETS expression system in UET-13 cells. CMV, cytomegalovirus. (B) Analyses for confirming the inducible expression of EWS/ETS genes. EWS/ETS mRNAs were detected in UET-13 transfectants UET-13TR-EWS/FLI1 and UET-13TR-EWS/ERG by RT-PCR. These cells were treated with or without 3 μg/ml of tetracycline (Tet) for the indicated periods. As an internal control, a human GAPDH gene was used. (C) Analyses for confirming the inducible expression of EWS/ETS proteins. The cells were treated as described for panel B and subjected to Western blotting for the detection of EWS/ETS proteins. The extracts of RD-ES and W-ES cells were also examined as positive controls. Membranes were reprobed with anti-actin antibody as a loading control. (D) Morphological change after tetracycline treatment of UET-13 cells and the transfectants were cultured in the absence or presence of tetracycline for 72 h and observed by light microscopy. Magnification, ×40 (top); ×200 (bottom). Cells were also examined using hematoxylin-eosin (HE) (top) and May-Giemsa (bottom) staining (magnification, ×200).

Real-time RT-PCR. Real-time RT-PCR was performed using TaqMan universal PCR master mix and TaqMan gene expression assays and an inventoried assay on an ABI Prism 7900HT sequence detection system (Applied Biosystems) according to the manufacturer's instructions. The human GAPDH gene was used as an internal control for normalization.

DNA microarray analysis. Total RNA isolated from cells was reverse transcribed and labeled using one-cycle target labeling and control reagents as instructed by the manufacturer (Affymetrix). The labeled probes were hybridized to the human genome U133 Plus 2.0 array (Affymetrix). The arrays were performed in a single experiment and analyzed using GeneChip operating software, version 1.2 (Affymetrix). Background subtraction, normalization, and principal component analysis (PCA) were performed by GeneSpring GX 7.3 software (Agilent Technologies). Signal intensities were prenormalized based on the median of all measurements on that chip. To account for the difference in detection efficiencies between the spots, prenormalized signal intensities on each gene were normalized to the median of prenormalized measurements for that gene. The data were filtered using the following steps. (i) Genes that were scored as absent in all samples were eliminated. (ii) Genes for which the signal intensities were lower than 100 were eliminated. (iii) Performing cluster analysis using filtering genes, we selected the genes that exhibited increased expression or decreased expression in tetracycline-treated cells. Accession numbers for the microarray data are given below.

Invasion assay. The invasion assay was performed using Matrigel (BD Bioscience) according to the previous description (34) with some modification. Polycarbonate filter inserts containing 8-μm pores (BD Falcon) were coated with 50 μl of a 6:1 mixture of culture medium and Matrigel and placed into 24-well culture plates containing DMEM supplemented with 10% T-FBS as chemoattractants. Cells (2.5×10^4) treated with or without tetracycline for 72 h were suspended in DMEM containing 0.01% T-FBS and plated on top of each filter insert. After 20 h in culture in the presence or absence of tetracycline, noninvading cells were removed from upper surface of the filter with a cotton swab. The invading cells on the lower surface of the filter were fixed with formalin, stained with hematoxylin-eosin, and counted in five fields per membrane with light microscopy. As a control, cells were also cultured on uncoated filter inserts. The invasion efficiency was presented as the ratio of the number of invading cells on Matrigel-coated inserts to that on uncoated inserts. Experiments were performed in triplicate, and the means with standard deviations of the values are shown in the graphs in Fig. 8.