Underexpression of RhoH in Hairy Cell Leukemia

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Abstract

The cause of hairy cell leukemia (HCL) is unknown. Current treatments seem effective only for a limited period of time. In addition, a significant proportion of patients remain refractory to all treatment options. These considerations indicate the need to develop alternative therapeutic strategies for HCL. Here, we report that HCL is characterized by underexpression of RhoH. In vitro reconstitution of RhoH expression inhibits the aberrant adhesion and transendothelial migration that drives disease pathogenesis. In an in vivo model of HCL, RhoH reconstitution limits malignant progression and protects against mortality. These findings provide the proof of principle that RhoH reconstitution represents a potential new approach to the treatment of HCL. [Cancer Res 2008;68(12):4531–40]

Introduction

Hairy cell leukemia (HCL) is a chronic lymphoproliferative disease, the cause of which is unknown (1–3). The disease is characterized by pancytopenia, hepatomegaly, splenomegaly, leukocytosis, and neoplastic mononuclear cells in the peripheral blood, bone marrow, liver, and spleen (1–3). Purine analogues are the current treatments of choice (2, 4, 5). However, a significant proportion of HCL patients remain refractive to these compounds (4, 5). In addition, disturbing new evidence that shows purine analogues only delay disease progression rather than provide a cure has emerged (6). HCL is a disease of late middle age (2, 3). Consequently, in countries with aging populations, HCL is set to become an increasing health care burden. These considerations highlight the need to develop alternative approaches to the treatment of HCL.

The malignant cells of HCL are diagnosed biochemically by aberrant expression of tartrate resistant acid phosphatase and the β2-integrin CD11c (7–12). Critical to the expression of the CD11c gene is its interaction with the transcription factor activator protein-1 (AP-1) (13). Normally, AP-1 expression is transient and mediates induction of CD11c in response to specific differentiation signals (14). However, in the neoplastic lymphocytes of HCL, AP-1 is expressed constitutively, thus driving abnormal concomitant expression of CD11c (13). One of the most important mechanisms controlling AP-1 expression is protein phosphorylation regulated by the Ras family of proto-oncogenes (15–25). In previous studies, we determined that, in HCL, Ras activation drives chronic expression of AP-1 that in turn drives the abnormal expression of CD11c diagnostic of the disease. Here, we report that, in addition to abnormal overexpression of AP-1 and Ras, HCL is also characterized by abnormal underexpression of RhoH (26, 27). This molecule is expressed exclusively by hematopoietic cells and lacks GTPase activity. Due to its inability to hydrolyze GTP, RhoH remains GTP bound and so acts as a noncompetitive inhibitor of members of the Ras family that cycle between forms bound by GTP and GDP (28, 29). Reconstitution of RhoH expression reduces HCL proliferation and both homotypic and heterotypic adhesion in vitro. RhoH reconstitution also reduced the process of transendothelial migration that is central to HCL pathogenesis. Finally, in an in vivo xenograft mouse model of HCL, we found that RhoH reconstitution inhibited malignant progression and protected against mortality. These findings thus provide proof-of-principle that RhoH reconstitution represents a promising new therapeutic strategy to combat HCL.

Materials and Methods

Cell culture. The hairy cell line Mo, the T-cell lines CEM and HSB-2, and the B-cell lines Raji, Namalwa, Daudi, and U-266 were obtained from the American Type Culture Collection and grown according to their specifications (30–35). The B-cell line VAL was provided by Dr. Christian Bastard (Laboratoire de Genetique Oncologique, Centre Henri Becquereil; ref. 36). The Karpass 231 B-cell line was provided by Dr. Abraham Karpass (University of Cambridge; ref. 37). The hairy cell line HC-1 was obtained from the Deutsche Sammlung von Mikroorganis men und Zellkulturen GmbH and grown according to their specifications (38). The hairy cell line EH was provided by Dr. Guy B. Faguet (Veterans Administration Medical Center; ref. 39). The hairy cell line ESKOL was provided by Edward F. Srour (Indiana University School of Medicine; ref. 40). The hairy cell lines JOK-1, Hair-M, and J-C-1 were provided by Jorn Koch (Aarhus University Hospital; ref. 41). Human microvascular endothelial cells (HMEC-1) were a gift of Dr. Sean P. Colgan (Brigham and Women's Hospital; ref. 42). CdCl2 was obtained from Fluka Chemie AG and used at concentrations of 1 or 10 μmol/L, where indicated. Lipopolysaccharide (LPS) was obtained from EMD Biosciences, Inc., and used at a final concentration of 100 ng/mL, where indicated.

Isolation of B-lymphocytes from human peripheral blood and spleens. After informed consent, peripheral blood was drawn from normal volunteers and patients with HCL. Also, after informed consent, spleens were removed from patients with chronic immune idiopathic thrombocytopenia (ITP) or splenic lymphoma with villous lymphocytes (SLVL). Subsequent morphologic examination indicated that the spleens of ITP patients were normal. Blood and spleen leukocytes were prepared by ficoll gradient centrifugation and frozen in liquid nitrogen. After thawing, cells were washed twice in RPMI 1640 containing 15% fetal bovine serum (FBS) and then resuspended in ice-cold PBS supplemented with 0.5% FBS and 2 mmol/L EDTA. B-lymphocytes were isolated by negative selection using the B Cell Isolation Kit II (Miltenyi Biotec). Cells isolated from HCL patients were determined to be over 90% positive for surface expression of CD19 and CD103 by flow cytometry. Cells isolated from normal individuals were over...
90% positive for CD19, but negative for CD103. Cells isolated from patients with SLVL or ITP were over 90% positive for CD19. Before RNA isolation, B-lymphocytes were washed once in PBS supplemented with 0.5% FBS but lacking EDTA.

**Plasmid construction.** The activity of the CD11c promoter was assessed by using the expression vector pATLuc that contains a promoterless firefly luciferase reporter gene (43). PCR was used to generate a fragment of the CD11c gene representing nucleotides -128 to +36 relative to the major transcription initiation site. This fragment was then subcloned into the "filled-in" HindIII site of pATLuc to generate p11c-Wt (13, 44). The ability of RhoH to repress the CD11c promoter was assessed using the expression constructs pCMV-RhoH and pMEP-RhoH. These were generated by first isolating the EcoRI/XbaI and KpnI/XbaI fragments of pCDNAs-VSVG-RhoH-Wt that encode RhoH tagged at its NH2 terminus with the vesicular stomatitis virus glycoprotein (VSVG) epitope. The isolated EcoRI/XbaI fragment was then cloned between the EcoRI and XbaI sites of pCDNA3.1zoe(+) (pCMV; Invitrogen Corp.) to produce pCMV-RhoH. The KpnI/XbaI fragment containing RhoH was cloned between the KpnI and NheI sites of pMEP4 (Invitrogen Corp.) to generate pMEP-RhoH. The activity of the RhoH gene promoter was assessed using the expression vector pGL4.14[Luc2/Hygro] that contains a promoterless firefly luciferase reporter gene (Promega Corp.). PCR was used to generate a fragment of the RhoH gene representing nucleotides -236 to +67 relative to a transcription initiation site used in B-lymphocytes. This fragment was then subcloned into the HindIII site of pGL4.14[Luc2/Hygro] to generate pGL4-RhoH. The transfection control plasmid p5-SV-βGal that directs expression of β-galactosidase was purchased from Promega Corp.

**RNA isolation.** Total RNA was isolated from cell lines and human B-lymphocytes by use of the High Pure RNA Isolation kit (Roche Diagnostics). The procedure included a DNase-I treatment to eliminate genomic DNA contamination. Amounts and quality of RNA were evaluated by UV spectroscopy using an Agilent 2100 Bioanalyzer (Agilent Technologies).

**PCRs.** The cDNA templates for quantitative reverse transcriptase–PCR (Q-RT-PCR) were prepared by random priming of 5 μg of total RNA with 200 units of Moloney murine leukemia virus reverse transcriptase in a final volume of 100 μL. The quality and quantity of templates were evaluated by analysis of ABL mRNA in accordance with the recommendations of Europe Against Cancer (45). The B-cell line Namalwa that expresses high levels of ABL and RhoH mRNA was used to produce calibration curves that evaluated PCR efficiency. RhoH and ABL mRNA were measured separately using TaqMan technology (Applied Biosystems). Real-time quantitative PCR primer pairs and probes were chosen with the assistance of the computer program Primer Express (Applied Biosystems). Due to heterogeneity at the 5’ end of RhoH mRNA, care was taken to design PCR primers that amplified all RhoH transcripts (46). The sense primer used had the nucleotide sequence 5’-TTTGGAAACTTCTCCTTCACACAC-3’ and the antisense primer had the sequence 5’-GCCCATCAGACACGGCTT-3’. The size of the PCR product resulting from the use of these primers was 81 bp and, therefore, compatible with good amplification efficiency. The RhoH probe had the sequence 5’-ATGGTGAAGCTAGCTTTTACACAC-3’ and the antisense primer had the sequence 5’-GCCCATCAGACACGGCTT-3’. The size of the PCR product resulting from the use of these primers was 71 bp and, therefore, compatible with good amplification efficiency. The RhoH probe had the sequence 5’-ATGGTGAAGCTAGCTTTTACACAC-3’ and the antisense primer had the sequence 5’-GCCCATCAGACACGGCTT-3’. The size of the PCR product resulting from the use of these primers was 81 bp and, therefore, compatible with good amplification efficiency. The RhoH probe had the sequence 5’-ATGGTGAAGCTAGCTTTTACACAC-3’ and the antisense primer had the sequence 5’-GCCCATCAGACACGGCTT-3’. The size of the PCR product resulting from the use of these primers was 81 bp and, therefore, compatible with good amplification efficiency. The RhoH probe had the sequence 5’-ATGGTGAAGCTAGCTTTTACACAC-3’ and the antisense primer had the sequence 5’-GCCCATCAGACACGGCTT-3’. The size of the PCR product resulting from the use of these primers was 81 bp and, therefore, compatible with good amplification efficiency. The RhoH probe had the sequence 5’-ATGGTGAAGCTAGCTTTTACACAC-3’ and the antisense primer had the sequence 5’-GCCCATCAGACACGGCTT-3’. The size of the PCR product resulting from the use of these primers was 81 bp and, therefore, compatible with good amplification efficiency. The RhoH probe had the sequence 5’-ATGGTGAAGCTAGCTTTTACACAC-3’ and the antisense primer had the sequence 5’-GCCCATCAGACACGGCTT-3’. The size of the PCR product resulting from the use of these primers was 81 bp and, therefore, compatible with good amplification efficiency. The RhoH probe had the sequence 5’-ATGGTGAAGCTAGCTTTTACACAC-3’ and the antisense primer had the sequence 5’-GCCCATCAGACACGGCTT-3’. The size of the PCR product resulting from the use of these primers was 81 bp and, therefore, compatible with good amplification efficiency. The RhoH probe had the sequence 5’-ATGGTGAAGCTAGCTTTTACACAC-3’ and the antisense primer had the sequence 5’-GCCCATCAGACACGGCTT-3’. The size of the PCR product resulting from the use of these primers was 81 bp and, therefore, compatible with good amplification efficiency. The RhoH probe had the sequence 5’-ATGGTGAAGCTAGCTTTTACACAC-3’ and the antisense primer had the sequence 5’-GCCCATCAGACACGGCTT-3’. The size of the PCR product resulting from the use of these primers was 81 bp and, therefore, compatible with good amplification efficiency. The RhoH probe had the sequence 5’-ATGGTGAAGCTAGCTTTTACACAC-3’ and the antisense primer had the sequence 5’-GCCCATCAGACACGGCTT-3’.
RhoH is underexpressed in HCL mediated by a transcriptional mechanism. A, total RNA was extracted from the HCL cell lines Hair-M, Mo, EH, JOK-1, ESKOL, JC-1, and HC-1. Total RNA was also extracted from the cell lines CEM and HSB-2 representing T-acute lymphoblastic leukemia, the cell lines Raji, Daudi, and Namalwa representing Burkitt’s lymphoma, U-266 representing plasmocytoma, VAL representing B-cell non–Hodgkin’s lymphoma, and Karpas 231 representing de novo acute leukemia of mature B-cells. The quantitative levels of RhoH and ABL expression were determined for each individual cell line by Q-RT-PCR. The relative level of endogenous RhoH expression was then calculated by dividing the quantitative level of RhoH expression by the quantitative level of ABL expression.

Columns, mean of two independent experiments; bars, SE. B, top, agarose gel stained with ethidium bromide showing the products of RT-PCR reactions that evaluate the levels of RhoH and ABL mRNA expression in cell lines CEM (CM), HSB-2 (HS), Raji (RA), VAL (VA), U-266 (U2), Mo (MO), EH, ESKOL (ES), HC-1 (HC), JOK-1 (JO), and JC-1 (JC). NG, contains a PCR reaction performed with no cDNA template; M, contains DNA size markers. The size of these markers in base pairs is indicated on the left side of the figure. Bottom, total protein was extracted from the HCL B-cell lines JOK-1, EH, HC-1, and Hair-M and from the non-HCL B-cell lines Raji and Namalwa. 50 μg of EH protein, 100 μg of Namalwa, Raji, JOK-1, and Hair-M protein, and 150 μg of HC-1 protein were subject to SDS-PAGE followed by Western blotting. RhoH protein expression was analyzed using the rabbit polyclonal anti-RhoH antibody α-Rho-6005. Protein loading was checked by hybridizing the blots with an antitubulin antibody. Representative of two independent experiments. C, top, total RNA was extracted from the circulating B-lymphocytes of three normal individuals and from four patients with HCL. The relative levels of expression of RhoH mRNA were determined as in the top. D, a fragment of the RhoH gene representing nucleotides -236 to +67 relative to a transcription initiation site used in B-lymphocytes was cloned upstream of the luciferase gene in pGL4.14[Luc2/Hygro] to generate the construct pGL4-RhoH. The transfection control plasmid pSV-βGal was mixed with either the parental vector pGL4.14[Luc2/Hygro] or pGL4-RhoH. The two plasmid mixtures were then transfected into the HCL B-lymphocyte cell line EH or the non-HCL B-lymphocyte cell line Raji. The levels of β-galactosidase activity directed by pSV-βGal were taken as reflective of transfection efficiency and used to correct the luciferase assay results. After correction for transfection efficiency, the level of luciferase reporter gene activity directed by pGL4-RhoH above that conferred by pGL4.14[Luc2/Hygro] was calculated. Columns, mean of three independent experiments; bars, SE.
with a micrometric grid of 1 mm² divided into 100 squares of 0.01 mm². All measurements represented the average values obtained from six representative fields of 1 mm². The size of the aggregates was evaluated as a function of time by measurement of their surface area expressed in mm². Aggregates were classified into the three size categories of small (0.1–0.25 × 0.01 mm²), medium (0.5–1.0 × 0.01 mm²), and large (1.5–20.0 × 0.01 mm²). The percentage of aggregates in each category was then calculated using the formula: (number of clumps in a given category / total number of clumps) × 100. The percentage of cells that were not engaged in aggregates was also analyzed. In this case, measurements were averaged from 10 representative fields of 1 mm² and obtained by counting every cell not engaged in an aggregate. In parallel, 5 ml of the culture were removed; aggregates in this aliquot were disrupted by repeat pipetting, and the total number of cells was averaged from 10 representative fields of 1 mm². The percentage of nonaggregated cells was then calculated by dividing the average number of nonaggregated cells per 1 mm² field by the average total number of cells per 1 mm² field and multiplying this value by 100.

**Lymphocyte-endothelial adhesion assays.** Monolayers of the endothelial cell line HMEC-1 were seeded into 96-multwell culture plates at a density that a previous experiment using crystal violet staining had shown produces confluency after 48 h. The resulting confluent monolayers were either left untreated or activated for 18 h with 100 ng/ml of LPS. Pools of JOK-1 cells stably transfected with pMEP4 or pMEP-RhoH were incubated for 30 min at 37°C in the presence of 5 μmol/L BCECF-AM (Calbiochem, EMD Biosciences, Inc.) then washed thrice in HBSS. Labeled cells (10⁴) were added to each well containing activated or nonactivated monolayers, culture plates were then centrifuged for 4 min at 150 × g to affect uniform settling, and adhesion was allowed for 1 h at 37°C. Monolayers were then gently washed thrice in HBSS, and fluorescence intensity was measured at 485 nm using a Mithras LB 940 multimode reader (Berthold Technologies, GmbH & Co KG). All values were normalized by subtraction of the fluorescence intensity of monolayers incubated in buffer alone. Specific cell adhesion was assessed by subtracting the fluorescence intensities obtained from nonactivated monolayers from the intensities obtained using activated monolayers. Adhesion assays for each stable JOK-1 pool were performed a total of thrice at minimum in quadruplicate.

**Transendothelial migration assays.** Confluent monolayers of HMEC-1 were prepared in the same way as for leukocyte-endothelial adhesion assays but in Transwell migration chambers of 6.5-mm diameter and 5.0-μm pore size (Corning, Inc.). Transwell inserts were set in HMEC-1 medium carried in 24-well plates and either left untreated or activated for 18 h with 100 ng/ml of LPS. Next, 10⁵ JOK-1 cells either stably expressing pMEP4 or pMEP-RhoH were prepared in 150 μl of HMEC-1 medium without LPS and added to the monolayers inside the Transwell inserts. These inserts were again set into 24-well plates and incubated for 8 h. After this time, the HMEC-1 medium under the Transwell inserts was supplemented with 100 ng/ml LPS to act as a chemoattractant. The Transwell inserts were then incubated at 37°C for 16 h, and the JOK-1 cells that migrated through to the medium on their underside were collected and counted. Migration assays were performed in triplicate, a total of thrice for each stable JOK-1 pool cultured with either activated or quiescent HMEC-1.

**Xenograft mouse model.** Male SCID mice (CB17 PRKDC, Charles River Laboratories), age 7 wk, were injected i.p. with 10⁴ JOK-1 cells suspended in RPMI 1640 without FBS. The JOK-1 cells that were injected either stably expressed pMEP-RhoH or the empty vector pMEP4. Control mice were injected with the RPMI 1640 vehicle alone. During the next 4 wk, the health of the mice was monitored, and if death occurred, analysis was performed immediately. After four weeks, surviving animals were sacrificed and their peritoneal cavity opened for tumor analysis. When present, tumors were dissected, their size measured, and photographs were taken. Two tumors were analyzed by cytometry to confirm they consisted of JOK-1 cells expressing human CD19. Spleens were also removed from injected mice, photographed, and weighed. This organ was then dilacerated, and its cellular contents were collected by irrigation with PBS. Erythrocytes present in the collection were lysed, and the remaining cells were subjected to cytometry analysis using an antibody that specifically recognizes human CD19 or an isotype-matched control antibody.

**Results**

RhoH is specifically underexpressed in cell lines derived from HCL. Previous studies have shown that the Ras family of proto-oncogenes is abnormally active in HCL (13, 25). Because RhoH represents a natural inhibitor of Ras in the hematopoietic lineage, we investigated the possibility that HCL may be characterized by RhoH underexpression. Initially, to address this question, we used Q-RT-PCR to examine RhoH mRNA levels in a

![Figure 2. RhoH reconstitution in HCL represses CD11c promoter activity. A fragment of the CD11c gene representing nucleotides -128 to +36 relative to the major transcription initiation site was cloned upstream of the luciferase gene in pATLuc to generate p11c-Wt (13, 43, 44). EH, Mo, and JOK-1 cells were transfected in parallel with p11c-Wt and pATLuc, each mixed with the plasmid pRSV-β; that constitutively expresses β-galactosidase. Transfections of EH and Mo were performed in the presence of pCMV-RhoH that constitutively expresses RhoH or in the presence of its empty equivalent pCMV. Transfections of JOK-1 were performed in the presence of pMEP-RhoH, where RhoH is expressed from the Metallothionine promoter or the empty parental plasmid pMEP4. Transfected JOK-1 cells were cultured with 1 μmol/L CdCl₂, and transfected EH and Mo were cultured without CdCl₂ for 16 h before β-galactosidase and luciferase assays were performed. The levels of β-galactosidase activity were taken as reflective of transfection efficiency and used to correct the firefly luciferase assay results. After correction for transfection efficiency, the level of luciferase reporter gene activity directed by p11c-Wt above that conferred by pATLuc in the presence of pCMV or pMEP4 was assigned a value of 100% (-RhoH). The level of activity directed by p11c-Wt in parallel transfections in the presence of pCMV-RhoH or pMEP-RhoH is expressed as a percentage of this value (+RhoH). Columns, mean of three independent experiments; bars, SD.**
RhoH is underexpressed in neoplastic lymphocytes isolated directly from HCL patients. Because RhoH seems underexpressed in HCL cell lines, we next wanted to determine whether this represented an accurate reflection of the malignant cells circulating within the blood stream of HCL patients. Therefore, we isolated B-lymphocytes from the blood of three normal donors and from four patients with HCL. Examination by Q-RT-PCR showed that RhoH expression levels were, on average, 5.2-fold lower in the B-lymphocytes of HCL patients compared with normal individuals (Fig. 1C, top). In contrast to this substantial suppression of RhoH in HCL lymphocytes, splenic B-lymphocytes isolated from patients with SLVL exhibited, on average, only a 27% reduction in RhoH expression (Fig. 1C, bottom).

RhoH underexpression in HCL cells is mediated by transcriptional repression of the RhoH gene promoter. RhoH underexpression in HCL could be mediated by transcriptional and/or posttranscriptional events. We investigated the former possibility by isolating the human RhoH promoter spanning nucleotides −236 to +67 relative to a transcription initiation site used in B-lymphocytes (46). This promoter region was then cloned upstream of the luciferase reporter gene present in pGL4.14[Luc2/Hygro] to generate the construct pGL4-RhoH. When pGL4-RhoH was transfected into EH hairy cells, it directed expression only 2.1-fold above background that directed by pGL4.14[Luc2/Hygro] empty of RhoH gene sequences (Fig. 1D). In contrast, when pGL4-RhoH was transfected into the non–hairy cell B-lymphocyte cell line Raji, it directed expression 15.6-fold above background (Fig. 1D). Thus, the transcriptional activity of the wild-type RhoH promoter is 7.4-fold lower in HCL cells than in nonhairy cells. These results indicate that transcriptional repression of the RhoH gene promoter plays a major role in RhoH underexpression in HCL cells.

Transient reconstitution of RhoH expression represses the aberrant activity of the CD11c gene promoter that characterizes HCL. The β2-integrin CD11c is normally largely restricted in its expression pattern to cells of the myeloid lineage (11). Its abnormal expression on the surface of HCL lymphocytes is used as a diagnostic marker for the disease (9). In previous studies, we have shown that in HCL abnormal expression of the CD11c gene reflects abnormal transcription of the gene by which it is encoded (13). We have further shown that this abnormal transcription is Ras-dependent and driven by the promoter region of the CD11c gene extending from −128 to +36 relative to the major site of transcription initiation. Because high levels of Ras-dependent transcription from the CD11c promoter are characteristic of HCL, we sought to determine whether this might result from underexpression of RhoH. To test this possibility, we used the HCL cell lines Mo, EH, and JOK-1. A construct constitutively expressing range of cell lines derived from both patients with HCL and other forms of leukemia. This study indicated that, on average, the relative expression of RhoH mRNA in HCL cell lines was 3.4-fold lower than that in cell lines derived from Burkitt’s lymphoma (Raji, Daudi, and Namalwa), plasmocytoma (U-266), T-acute lymphoblastic leukemia (HSB-2 and CEM), de novo acute B-cell leukemia (Karpas 231), and B-cell non–Hodgkin’s lymphoma (VAL; Fig. 1A and B, top). In addition to mRNA levels, RhoH protein levels were also significantly repressed in HCL cell lines compared with non-HCL cells (Fig. 1B, bottom). Specifically, we found RhoH protein to be undetectable in the HCL cell lines HC-1 and Hair-M and reduced 2.7-fold and 3.3-fold in EH and JOK-1, respectively, compared with its average expression in the Burkitt’s lymphoma lines Raji and Namalwa.

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**Figure 3.** RhoH reconstitution reduces surface expression of CD11c. Pools of JOK-1 cells were generated that contain within their genome either pMEP4 or pMEP-RhoH. A, agarose gel stained with ethidium bromide after electrophoresis of RT-PCR products generated from JOK-1 cells expressing pMEP4 or pMEP-RhoH. The pairs of oligonucleotides used in the PCR generated products from mRNA encoding recombinant RhoH tagged with the VSVG epitope (RhoH) or from the ABL gene product (ABL). The oligonucleotides fail to generate products from endogenous RhoH mRNA. Lane 1, size markers; lane 2, RT-PCR product using distilled water as template; lane 3, RT-PCR product using as template mRNA isolated from JOK-1 carrying pMEP4 and cultured in the absence of supplementary CdCl2; lane 4, JOK-1 carrying pMEP4 and cultured in the presence of 10 μmol/L supplementary CdCl2; lane 5, JOK-1 carrying pMEP-RhoH and cultured in the absence of supplementary CdCl2; lane 6, JOK-1 carrying pMEP-RhoH and cultured for 24 h with 1 μmol/L supplementary CdCl2; lane 7, JOK-1 carrying pMEP-RhoH and cultured for 48 h with 1 μmol/L supplementary CdCl2; lane 8, JOK-1 carrying pMEP-RhoH and cultured for 24 h with 10 μmol/L supplementary CdCl2; lane 9, JOK-1 carrying pMEP-RhoH and cultured for 48 h with 10 μmol/L supplementary CdCl2. This analysis showed that cells carrying pMEP4 expressed no recombinant RhoH whereas cells carrying pMEP-RhoH produced recombinant RhoH either in the presence or absence of supplementary CdCl2. B, Western blots of total protein extracted from parental JOK-1 cells (Parent) or JOK-1 stably expressing pMEP-RhoH (RhoH). Both cell lines were cultured in the absence of supplementary CdCl2. Expression of RhoH was analyzed using the antibody α-Rho-6005 and protein loading analyzed with an antibody that recognizes tubulin. Representative of two independent experiments. C, flow cytometric analysis was performed on JOK-1 stably expressing pMEP4 (Empty) and on JOK-1 cells stably expressing pMEP-RhoH (RhoH). Both cell lines were cultured in the absence of supplementary CdCl2, and cytometry was performed using a monoclonal antibody that specifically binds CD11c (CD11c Ab) or an isotype matched control (Iso-Match Ab). The percentages of cells that exhibit fluorescence in a defined range of intensity are presented from a representative of three independent experiments. The mean reduction in CD11c expression determined from these experiments was 49.5% ± SD of 9%.

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human RhoH was transfected into these cell lines in combination with either a CD11c promoter construct or its parental vector empty of CD11c sequences. In Mo, EH, and JOK-1, the expression of exogenous RhoH caused, on average, 66%, 66%, and 52% reductions, respectively, in the transcriptional activity of the CD11c promoter (Fig. 2). These results showed that RhoH repression contributes to the expression of the CD11c promoter in hairy cells.

Stable reconstitution of RhoH expression reduces cell surface expression of CD11c protein. In JOK-1 hairy cells, transient reconstitution of RhoH expression represses the CD11c gene promoter when the promoter is present in an extrachromosomal plasmid (Fig. 2). Next, we investigated the possibility that RhoH reconstitution might also cause a reduction in the surface expression of the CD11c protein, as this would reflect repression of the endogenous CD11c gene. Pools of JOK-1 that contain genomic integration of either an empty expression plasmid or this same plasmid constitutively expressing RhoH were generated (Fig. 3A and B). The surface expression of CD11c in these two pools of cells was then assessed using flow cytometry. This analysis established that stable reconstitution of RhoH expression causes, on average, a 49.5% decrease in CD11c expression (Fig. 3C). Consequently, in JOK-1 cells, reconstitution inhibits endogenous, as well as exogenous, CD11c expression.

Reconstitution of RhoH expression inhibits the homotypic adhesion of HCL. One of the hallmarks of HCL is the extravasation of neoplastic lymphocytes (1–3). Such extravasation is dependent upon malignant cells exhibiting increased intercellular adhesion. In cell culture, this increased capacity for adhesion is manifest by HCL cell lines typically growing in large clumps. We have shown that RhoH reconstitution in HCL reduces expression of the broadhesion molecule CD11c. Others have shown that RhoH holds the CD11a/CD18 heterodimer in an inactive conformation (47). Because RhoH seems to inhibit functional expression of molecules that mediate leukocyte adhesion, we tested the hypothesis that RhoH reconstitution in HCL might inhibit homotypic intercellular adhesion. The pools of JOK-1 that either stably express RhoH or an empty vector were disrupted into cultures of single cells and then incubated undisturbed for 3 days (Fig. 4A). After this period of time, we found that, compared with the pools expressing the empty vector, the pools expressing RhoH had over 5-fold fewer cells growing as aggregates (Fig. 4B). In

Figure 4. Reconstitution of RhoH expression in HCL inhibits homotypic adhesion. A, light microscope images of JOK-1 cells that contain in their genome either pMEP4 (Empty) or pMEP-RhoH (RhoH). Cells were photographed 3 d after a culture of single cells was initiated in the absence of supplementary CdCl2. B, the percentage of cells not present in an aggregate of two or more. These values were calculated each day for 3 d after single-cell cultures were initiated from 0.5 × 10^6 JOK-1 cells that stably express either pMEP4 or pMEP-RhoH. Columns, mean of 10 microscopic fields of 1 mm^2 of two independent experiments; bars, SE. C, the percentage of cells present in aggregates classified as small (S; 0.1–0.25 × 0.01 mm^2), medium (M; 0.5–1.0 × 0.01 mm^2), or large (L; 1.5–20.0 × 0.01 mm^2). These values were calculated each day for 3 d after single-cell cultures were initiated. Columns, mean of six microscope fields of 1 mm^2 of two independent experiments; bars, SE. Asterisks denote values of 0%.
addition, the sizes of the aggregates that did exist were significantly reduced (Fig. 4C).

Reconstitution of RhoH expression inhibits the heterotypic adhesion of HCL. Our study of aggregation indicated that RhoH reconstitution in HCL cells inhibits their homotypic adhesion (Fig. 4). However, central to the process of HCL extravasation is the heterotypic adhesion of malignant cells to vascular endothelium. The ability of RhoH reconstitution to inhibit this kind of adhesion was assessed in vitro using a fluorescence assay we have previously used (48). Use of this assay showed that the pools of JOK-1 hairy cells reconstituted with RhoH exhibit a 58% reduction in adhesion to activated endothelial cells compared with control cells (Fig. 5A).

Reconstitution of RhoH expression inhibits HCL transendothelial migration. In addition to simple endothelial adhesion, HCL extravasation is dependent upon malignant cells being able to migrate through endothelium. Consequently, using Transwell migration chambers, we assessed the ability of RhoH reconstitution to inhibit HCL transendothelial migration. Equal numbers of control JOK-1 pools and pools reconstituted with RhoH were settled for 8 hours on top of quiescent or activated endothelial monolayers. JOK-1 migration was then elicited by adding LPS to the culture medium present below the endothelial monolayer, which was supplemented with 100 ng/mL LPS to act as a chemoattractant for the JOK-1 cells. After 16 hours, the JOK-1 cells that migrated through the support membrane of the endothelial monolayer were collected and counted. Columns, mean of three independent experiments; bars, SD.

Reconstitution of RhoH expression reduces HCL proliferation. Abnormal intercellular adhesion that effects transendothelial...
migration represents one of the pathologic hallmarks of HCL. The finding that such processes are dependent upon underexpression of RhoH suggests that reconstitution of RhoH may represent a useful therapeutic strategy. We further assessed this possibility by determining the ability of RhoH reconstitution to block HCL proliferation. Cultures were initiated from equal numbers of the JOK-1 pools that contained stable integration of an empty vector or this same vector expressing RhoH. The two pools were cultured in parallel, and after 0, 2, 4, 6, and 8 days, the cells were counted. The number of cells containing the empty vector was assigned a value of 100%, and the number of cells containing the RhoH plasmid was calculated as a percentage of this value. The results depicted in

Figure 6. RhoH reconstitution inhibits malignant progression in vivo. The peritoneum of SCID mice was injected with RPMI1640, RPMI1640 containing JOK-1 cells stably expressing the empty vector pMEP4, or RPMI1640 containing JOK-1 cells stably expressing the RhoH expression construct pMEP-RhoH. After 28 d, all surviving mice were sacrificed and the injection site and/or spleens were examined. A, top, seven mice that received cells expressing pMEP4 and eight mice that received cells expressing pMEP-RhoH were classified depending upon whether they had no visible tumor (NG), a small tumor of <1 cm in maximum diameter (S), a medium sized tumor of 1 to 1.4 cm (M), a large tumor of 1.5 to 2.4 cm (L), or a very large tumor of 2.5 to 3.5 cm (VL). Bottom, photographs of representatives of each tumor class. The smallest division of the ruler depicted in each image is 1 mm. B, top, weight of the spleens isolated from mice injected with RPMI 1640 alone (Control) or mice injected with JOK-1 cells expressing either pMEP4 (Empty) or pMEP-RhoH (RhoH). Columns, mean weight of 4, 9, and 12 mice, respectively; bars, SE. Bottom, representatives of spleens isolated from mice injected with JOK-1 cells expressing either pMEP4 (Empty) or pMEP-RhoH (RhoH). The smallest division of the ruler depicted in each image is 1 mm. C, a representative of cytometric analysis of human CD19 expression in spleen B-lymphocytes isolated from nine mice injected with JOK-1 cells expressing pMEP4 (Empty) or 12 mice expressing pMEP-RhoH (RhoH). Reactivity with the antihuman CD19 antibody that detects injected JOK-1 cells is indicated by the open line. This line is overlaid with a filled line indicating reactivity with an isotype-matched negative control antibody.
Fig. 5C indicate that RhoH reconstitution causes a reduction in HCL proliferation, such that after 8 days, the number of JOK-1 cells was reduced, on average, by 57%.

**RhoH reconstitution protects against malignant progression in vivo.** Our data generated in vitro show that RhoH reconstitution inhibits both the proliferation and adhesion of HCL cells. Next, we used a xenograft mouse model to determine whether RhoH reconstitution inhibits malignant progression in vivo. The JOK-1 pools that contained stable integration of an empty vector or this same vector expressing RhoH were injected into the peritoneum of SCID mice. Twelve mice were injected with each pool. During the course of 28 days, all mice injected with cells reconstituted with RhoH remained alive and vigorous. In contrast, all mice injected with nonreconstituted cells became increasingly sluggish with three dying at day 27. After 28 days, all surviving animals, including the vigorous subjects injected with RhoH reconstituted cells were sacrificed. The peritoneal cavity was opened, and the site of injection was examined in seven recipients of nonreconstituted cells and eight recipients of reconstituted cells. In the eight examined recipients of HCL cells reconstituted with RhoH, three failed to develop any manifestation of malignancy, two developed tumors of <1 cm in maximum dimension, and the other three had tumors of maximum dimension of 1.2, 1.4, and 1.8 cm. These observations were in striking contrast to mice injected with HCL cells not reconstituted with RhoH expression. Here, the seven mice examined all developed tumors of at least 2.1 cm maximum dimension, and two had tumors of a maximum dimension over 3 cm (Fig. 6A). Taken together, these data generated using mice indicate that reconstituting RhoH expression reduced HCL tumor burden by 70%. Further analysis indicated that RhoH reconstitution eliminated splenomegaly (Fig. 6B). In addition, the percentage of JOK-1 cells present in the spleen was reduced from 1.6% to 0% (Fig. 6C).

**Discussion**

We report here that HCL lymphocytes exhibit constitutive underexpression of RhoH mediated by repression of its gene promoter. Reconstitution of RhoH expression in vitro inhibits HCL proliferation, adhesion, and transendothelial migration. Use of a xenograft mouse model indicates that RhoH reconstitution limits malignant progression in vivo.

Cell proliferation is driven by Ras activation (49). Because RhoH is a negative regulator of the Ras family, this provides a molecular explanation for RhoH reconstitution being able to inhibit HCL proliferation. The ability of RhoH reconstitution to inhibit the transendothelial migration that results from HCL exhibiting increased intercellular adhesion can be explained in two ways. First, RhoH has been shown to hold the adhesion receptor CD11a/CD18 in an inactive form (47). Second, RhoH reconstitution is able to inhibit transcription of the gene that encodes the leukocyte adhesion molecule CD11c (Fig. 2). This inhibition is likely effected by inhibiting ras-signaling cascades. Such cascades we have shown to be abnormally activated in HCL leading to constitutive expression of the transcription factor AP-1 and subsequent induction of CD11c gene transcription.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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