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ORIGINAL ARTICLE

Modulation of B-cell receptor and microenvironment signaling by a guanine exchange factor in B-cell malignancies

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ABSTRACT

Objective: Chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL) cells over-express a guanine exchange factor (GEF), Rasgrf-1. This GEF increases active Ras as it catalyzes the removal of GDP from Ras so that GTP can bind and activate Ras. This study aims to study the mechanism of action of Rasgrf-1 in B-cell malignancies.

Methods: N-terminus truncated Rasgrf-1 variants have a higher GEF activity as compared to the full-length transcript therefore a MCL cell line with stable over-expression of truncated Rasgrf-1 was established. The B-cell receptor (BCR) and chemokine signaling pathways were compared in the Rasgrf-1 over-expressing and a control transfected cell line.

Results: Cells over-expressing truncated form of Rasgrf-1 have a higher proliferative rate as compared to control transfected cells. BCR was activated by lower concentrations of anti-IgM antibody in Rasgrf-1 over-expressing cells as compared to control cells indicating that these cells are more sensitive to BCR signaling. BCR signaling also phosphorylates Rasgrf-1 that further increases its GEF function and amplifies BCR signaling. This activation of Rasgrf-1 in over-expressing cells resulted in a higher expression of phospho-ERK, AKT, BTK and PKC-alpha as compared to control cells. Besides BCR, Rasgrf-1 over-expressing cells were also more sensitive to microenvironment stimuli as determined by resistance to apoptosis, chemotaxis and ERK pathway activation.

Conclusions: This GEF protein sensitizes B-cells to BCR and chemokine mediated signaling and also upregulates a number of other signaling pathways which promotes growth and survival of these cells.

KEYWORDS B-cell malignancies; mantle cell lymphoma; chronic lymphocytic leukemia (CLL); B-cell receptor; guanine exchange factor; Rasgrf-1; ERK pathway

Introduction

B-cell receptor (BCR) signaling and microenvironment signaling are the two important drivers of B-cell malignancies[1-4]. Signaling via these pathways activates extracellular signal-regulated kinase (ERK) pathway in addition to other signaling pathways. ERK pathway is upregulated in B-cell malignancies and its activation suppresses apoptosis, increases cell proliferation, and allows cells to become resistant to chemotherapy agents[1-8]. Inhibition of this pathway by a number of inhibitors induces apoptosis in these cells indicating that ERK pathway activation is required for survival of these leukemic cells[9].

Signaling for the ERK pathway is initiated by membrane receptor kinases which activate Ras that results in the activation of Raf/MEK/ERK followed by nuclear translocation of ERK. Ras activation is controlled by regulatory proteins, guanine exchange factors (GEFs), that promote active-GTP bound state, and GTPase activating proteins (GAPs) that return the GTPase to its GDP-bound inactive state[10]. Rasgrf-1 is an example of a GEF protein that stimulates the production of the active-GTP bound Ras and is over-expressed in B-cell malignancies [11-13]. Rasgrf-1 and other GEF proteins have transforming activity in cell-culture assays, demonstrate cooperativity with Ras and a number of RhoGEFs and RasGEFs have been identified as oncogenes[11,12]. Recent reports highlight the central role GEFs and GAPs as their activity is itself regulated by cytokines, adhesion receptors and G-protein coupled receptors which in turn alter ERK signaling (reviewed in 14). GEFs have a role in T- and B-cell receptor signaling pathways as GEFs such as Vav and Rasgrp1 function as downstream effectors of this signaling pathways[15-17]. GEFs harbor regulatory/inhibitory domains in the N-terminus regions and deletion of these auto-regulatory domains by protein truncation is known to activate their GEF function[18].

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BCR signaling is the major signaling pathway that defines clinical, biologic and prognostic characteristics of chronic lymphocytic leukemia (CLL)[1-2,5,19-22]. The activation or cross-linking of BCR results in the formation of an initial signaling complex (signalosome) that includes Lyn, Syk, BTK (Bruton tyrosine kinase), and Zap-70 among other components. This stimulates a number of downstream pathways such as PI3Kinase, PKC, NF-κB and Ras/Raf/MEK/ERK that result in cell proliferation, resistance to apoptosis, cell motility, migration etc. BCR signaling increases expression of anti-apoptotic genes such as Mcl1, XIAP and decreases expression of pro-apoptotic genes such as Bim and Bax, thus inhibiting cell apoptosis. Our previous studies have shown that BCR activation also phosphorylates Rasgrf-1 at residue Ser 929 that further increases its GEF function and increases Ras-GTP in leukemic cells[13].

Besides BCR signaling, microenvironment is the other equally important signaling component that drives B-cell malignancies. Bone marrow stromal cells (BMSC) and nurse like cells (NLC) provide growth factor and contact support to B-cell malignancies including CLL cells that leads to a higher proliferative rate and resistance to chemotherapy induced apoptosis[3,4,23-27]. Co-culture of CLL cells with BMSC enhances their survival as these cells provide growth factor and chemokine support along with signaling via integrins and the Notch pathway[28,29]. CXCL12 (SDF-1) and CXCL13 are two chemokines that activate CLL chemotaxis, actin polymerization and tissue homing[25]. Binding of chemokine, SDF-1 to its cognate receptor CXCR4 results in G-protein signaling, phosphorylation of ERK, chemotaxis and resistance to apoptosis[3,25].

As both BCR and microenvironment signals activate ERK, Rasgrf-1 over-expression could potentially regulate both BCR and microenvironment signaling pathways and result in Ras/Raf/MEK/ERK activation. Further characterization of these signaling pathways is of interest to define therapeutic targets that block this pro-survival signaling mechanism. To study this further, experiments were designed investigate the mechanism of action of Rasgrf-1. The role of this GEF protein was analyzed by establishing a stable mantle cell lymphoma (MCL) cell line with a N-terminus truncated Rasgrf-1 construct. These Rasgrf-1 over-expressing cells were analyzed for any alterations in BCR and microenvironment signaling.

Materials and methods

Cell culture and reagents

CLL specimens were obtained from patients at the West Los Angeles VA Hospital Hematology clinic after informed consent and Institutional Review Board approval. CLL specimens for this study were obtained from patients that had not received any prior treatment and had more than 90% CLL cells in the peripheral blood mononuclear cells (PBMC) isolated. Primary CLL cells were isolated by a ficoll gradient and stored in liquid nitrogen. Maver-1 cell line was obtained from ATCC[30]. Activation of BCR was performed by cross linking with a goat F(ab')2 anti-human IgM antibody (SouthernBiotech, Birmingham, AL) at a concentration of 10 mg/mL for 20 min or as indicated. Cells were also activated by co-culture with stromal cell line HS-5 cells (ATCC) for 24 h[31]. All cell culture experiments were performed with RPMI media with 10% FBS, glutamine 2mM, Pen-Strep, Sodium pyruvate 1mM.

Plasmid constructs and transfection

Full length human Rasgrf-1 cDNA (3822bp, 1273AA) was obtained from Origene, MA. With PCR, a 2004 bp fragment with intact 3' end of the gene was amplified from the cDNA (nucleotide 2194 to nucleotide 4197, 667AA NM_002891.4). This fragment was cloned in the pCDNA 3.1V5His Topo TA vector (Invitrogen, CA) in frame with the V5 epitope (Rasgrf-1rN605) and lacks 605 AA from the N-terminus region. A control vector with no insert was also constructed. Maver-1 cell line was transfected with Amaxa Nucleofector system using Solution V and program U07. Transfected cells were selected with G418 at 400 mg/mL and pools of selected cells were analyzed for further experiments.

Western blot analysis

Lysates were prepared by washing cells with cold PBS and disrupted in lysis buffer (Cell Signaling, MA) supplemented with protease inhibitor cocktail. Insoluble material was removed by centrifugation (10,000 *g*, 10 min) and protein concentrations determined by BioRad DC protein assay. Samples were mixed with SDS sample buffer and 20-30 μg aliquots resolved on SDS/PAGE gels. Rasgrf-1 antibody (C-terminus) was purchased from Proteintech (Chicago, IL), phospho-Rasgrf1 antibody (Serine 929) from Santa Cruz Biotechnology (Dallas, TX), phospho-ERK (Thr202/Tyr204), phospho-Akt (Ser473), phospho-BTK (Tyr223), and actin

from Cell Signaling (Beverly, MA). Detection was performed with horseradish peroxidase-conjugated secondary antibodies and chemiluminescence (ECL plus, GE Healthcare and LAS Mini imager, Fuji).

Apoptosis and chemotaxis assays

Apoptosis was analyzed by BD Annexin V FITC flow cytometry assay (Becton Dickinson). Briefly, 2×10^5 cells in a 6-well dish were treated with bendamustine at different concentrations and 48 h later stained and analyzed as protocol. Apoptosis was also analyzed in different co-culture conditions, with and without HS-5 co-culture. Cells were then stained and processed by the Annexin flow cytometry protocol. The chemotaxis assay was performed in Transwell culture plates (Costar, Cambridge, MA) with a pore size of 5 μm. Briefly, B-cell lines were suspended in RPMI-1640 with 0.5% BSA. A total of 100 μL, containing 10^5 cells, was added to the top chamber of Transwell culture inserts. Filters then were transferred to wells containing medium with or without SDF-1 (200 ng/mL, R&D systems). The chambers were incubated for 4 h at 37°C in 5% CO_2. After this incubation, the cells in the lower chamber were counted by running through a Accuri flow cytometer at 10 μL/min in triplicates.

Results

Rasgrf-1 over-expression in Maver-1 cell line

A MCL cell line was selected as a model to study the mechanism of action of this GEF protein in B-cell malignancies. MCL cells share a number of cell surface markers with CLL and have a functional BCR similar to CLL that is important for cell signaling. MCL lines can be propagated and are readily transfectable as compared to primary CLL specimens. In Rasgrf-1, the N-terminus domains are inhibitory to the GEF function of this protein therefore a number of tumor cell lines express shorter versions of Rasgrf-1 that have intact C-terminus and a higher GEF activity [18]. To replicate this observation a N-terminus truncated Rasgrf-1 expression vector was designed to include the C-terminus region of Rasgrf-1 (Rasgrf-1rN605)(**Figure 1A**). Maver-1 cells were transfected with this expression vector and pools of stably selected cells were further analyzed.

To detect ectopic expression of Rasgrf-1 Western blots were performed and an additional band of Rasgrf-1 (80kD) was observed (**Figure 1B**). This blot also confirms that this cell line expresses a number of smaller Rasgrf-1 isoforms and lacks the expected 140kD protein. The anti-Rasgrf-1 antibody is against the C-terminal region of the protein and all these isoforms thereby harbor the C-terminus GEF domain that is functionally important for Rasgrf-1 activity. This 80 kd additional band is not present in the control transfected cells and corresponds to the band observed in the Western blot performed with the anti-V5 antibody (**Figure 1B** lower panel). To analyze the growth characteristics of the Maver-1 Rasgrf-1 cells a growth curve assay was performed. A total of 10^4 cells of Maver-1 Rasgrf-1rN605 and Maver-1 Neo (control transfection) were plated in 6-well dish and viable cell count was determined at different time intervals. **Figure 1C** shows that ectopic expression of truncated Rasgrf-1 in this cell line increases the growth rate of these cells. Activation of Ras by this GEF thus further increases the proliferative potential of these cells.

Effect of Rasgrf-1 on BCR signaling

BCR signaling is the single most important pathway in B-cell malignancies. Increase in the expression of a GEF protein can potentially enhance signaling in these cells including an upregulation of Ras/Raf/MEK/ERK pathway. To study this we activated BCR signaling via IgM cross-linking by different concentrations of the anti-IgM antibody. As Rasgrf-1 could potentially amplify BCR responses, it is possible that the cell lines exhibit different sensitivity for BCR activation. **Figure 2A** and **B** show the phosphorylation of ERK when the two cell lines, Maver-1 Rasgrf-1rN605 and Maver-1 Neo were cross-linked at different concentrations of anti-IgM antibody. In the Maver-1 Neo cell line ERK phosphorylation is observed at a concentration of 10 mg/mL of anti-IgM antibody, a concentration that is used for cross-linking experiments[13]. However the Maver-1 Rasgrf-1rN605 cells activate their BCR signaling and ERK phosphorylation at much lower concentrations of anti-IgM antibody. In addition when these lysates were examined for Rasgrf-1 phosphorylation, a similar pattern is observed, i.e. phosphorylation of Rasgrf-1 at lower concentrations of anti-IgM antibody in Maver-1 Rasgrf-1rN605 cells. This phosphorylation of the GEF itself by BCR activation is important as it is known that phosphorylation of Rasgrf-1 increases its GEF function and is therefore a mechanism of amplifying BCR mediated responses.

Besides the Ras/Raf/MEK/ERK pathway, activation of other signaling pathways downstream of BCR was also analyzed in the two cell lines. Maver-1 Rasgrf-1rN605 and Maver-1 Neo cells were cross-linked with 10mg/mL of anti-IgM antibody for 20 min. Lysates were analyzed for phosphorylation of ERK, Akt[19], PKC-alpha[32] and BTK[33]

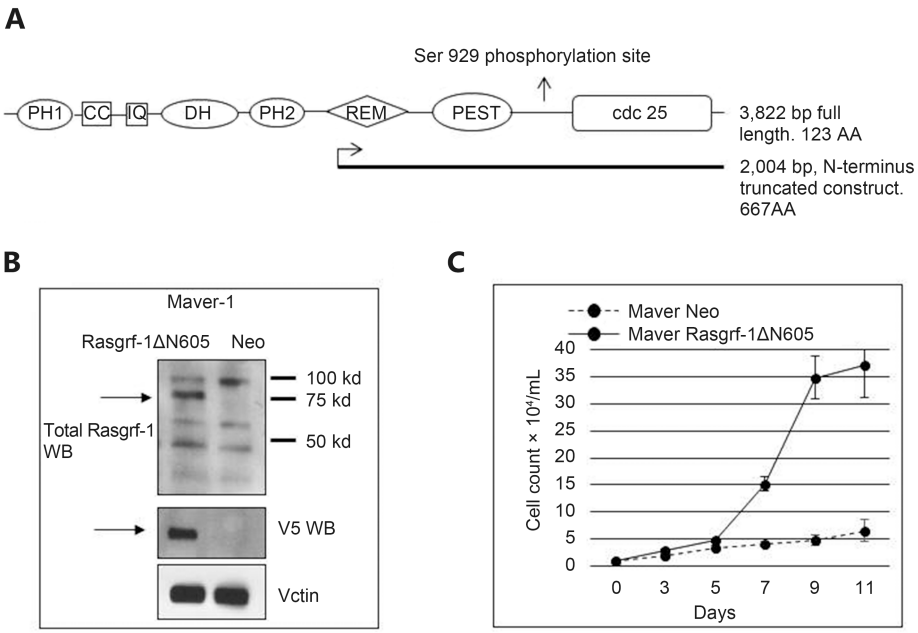

Figure 1 Rasgrf-1 RNA schematic and its expression in MCL cell line. (A) Domain structure of Rasgrf-1 (full length 1,273 amino acids). CDC 25 is the GEF domain, Ser 929 is the serine residue phosphorylated by BCR activation and microenvironment signaling. The lower line represents the truncated construct (Rasgrf-1rN605, 667 amino acids) with N-terminus deletion. (B) Stably transfected Maver-1, a mantle cell line with the truncated Rasgrf-1 construct. The truncated protein is expressed in-frame with a V5 epitope. Figure shows Western blots (upper) with total Rasgrf-1 antibody and the lower with anti-V5 epitope antibody. Maver-1 Neo (control) and Maver-1-Rasgrf-1rN605 stably transfected cells were analyzed with two different antibodies. Arrows on the right side indicate multiple Rasgrf-1 protein bands of different sizes expressed in the parent cell line. The arrow on the left side points towards the 80 kD additional band in the Maver-1-Rasgrf-1rN605 cells. Lower panel shows identical lysates probed with an anti-V5 epitope antibody and the expression of 80 kD protein band. (C) Growth curve for Maver-1 Neo and Maver-1 Rasgrf-1 expressing cell lines. Viable cell count performed on day 3, 5, 7, 9 and 11. Line graph shows data from two separate experiments (mean±SD).

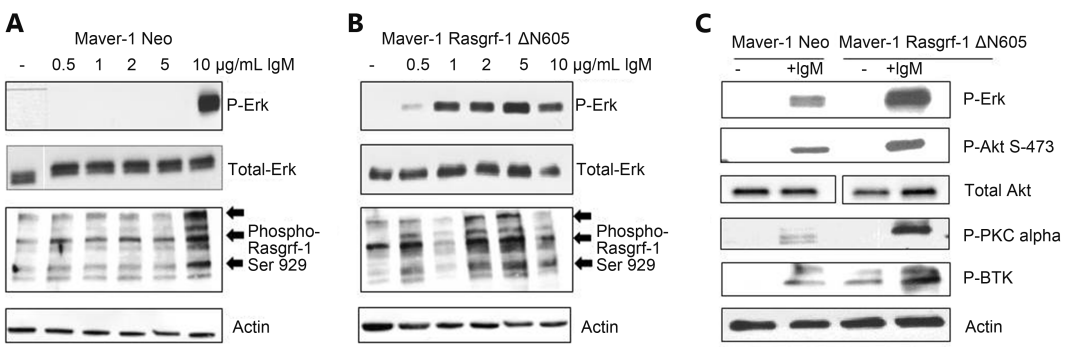

Figure 2 Modulation of BCR signaling by the GEF protein Rasgrf-1. (A) and (B) Maver-1 Neo and Maver-1-Rasgrf-1rN605 cell lines were treated with different concentrations of anti-IgM antibody (IgM) for twenty minutes and the lysates were analyzed for ERK. P-ERK (phospho-ERK), Phospho-Rasgrf-1 (Ser 929) and actin. (C) Maver-1 Neo and Maver-1-Rasgrf-1rN605 cell lines were stimulated with anti IgM antibody (10 mg/mL for 20 min) and lysates analyzed for P-ERK, P-Akt Ser 473, total Akt, phospho-PKC alpha and phospho-BTK signals by specific antibodies.

(**Figure 2C**) in Rasgrf-1rN605 expressing cells as compared to the control cells. ERK, PKC and Akt pathways are important mediators of BCR signaling and their activation in turn leads to resistance to apoptosis, cell proliferation and

chemotaxis. BTK on the other hand is a more proximal signaling component of the BCR, complexes with Syk and Lyn initiating downstream signaling. The results indicate that expression of this GEF leads to a higher activation of additional signaling pathways as determined by the phosphorylation of Akt, PKC-alpha and BTK. This GEF thus has the potential of affecting other important signaling pathways as well.

Effect of Rasgrf-1 expression in microenvironment signaling

The Ras/Raf/MEK/ERK pathway integrates a number of extracellular stimuli and activates ERK. As Ras signaling is amplified by its GEF, Rasgrf-1, experiments were performed to determine whether over-expression of this GEF (Rasgrf-1rN605) will enhance the microenvironment signaling in the B-cell leukemia model. To stimulate microenvironment signaling, cells were grown in a co-culture with HS-5, a HPV E6/E7 transformed stromal cell line. This cell line supports growth of hematopoietic cells by secreting a number of cytokines and chemokines including, IL-1, 6, 8, 11, GM-CSF, G-CSF etc. Maver-1 Neo and Maver-1 Rasgrf-1rN605were co-cultured with HS-5 cells for 24 h and then analyzed for Ras/Raf/MEK/ERK pathway activation by western blot analysis. There is a stronger phospho-ERK signal in the Rasgrf-1 over-expressing cells as compared to the control cells (**Figure 3A**). As shown in **Figure 2C**, there is no baseline phospho- ERK signal in these cell lines. In addition to BCR, the microenvironment signaling is an additional mechanism of activating the Ras/Raf/MEK/ERK pathway and the ectopic expression of Rasgrf-1 is further able to enhance this signaling.

Phosphorylation of Rasgrf-1 was analyzed (**Figure 3B**) by Western blots that demonstrate the phosphorylation of this GEF by microenvironment signaling. This is observed in both the cell lines and the ectopically expressed Rasgrf-1. It is also phosphorylated as an additional band in Maver-1 Rasgrf-1rN605 cells. There is a lower ERK phosphorylation signal in Maver neo cells even though the endogenous Rasgrf-1 is phosphorylated (**Figure 3B**). This can be explained in part by the role of additional signaling intermediates that may be involved in the ERK phosphorylation. It is possible that the higher phosphorylation of Akt, PKC-alpha and BTK in Maver-1 Rasgrf-1rN605 cells (**Figure 2C**) have a role in effective ERK phosphorylation in these cells. Our previous results demonstrate the phosphorylation of Rasgrf-1 in CLL specimens with BCR signaling[13], however phosphorylation with stromal cell co-culture stimulation is not known. To determine this, two primary CLL specimens were co-cultured with HS-5 cells and western blot performed with phospho-Rasgrf-1 antibody. **Figure 3C** shows that there is phosphorylation of Rasgrf-1 and ERK phosphorylation by stromal co-culture in these leukemic cells as well. This finding further highlights the role of this GEF as micro-environment signaling is increasingly recognized as being critical for growth and apoptotic resistance of leukemic cells.

Apoptosis and chemotaxis

Resistance to apoptosis is a hallmark of B-cell malignancies.

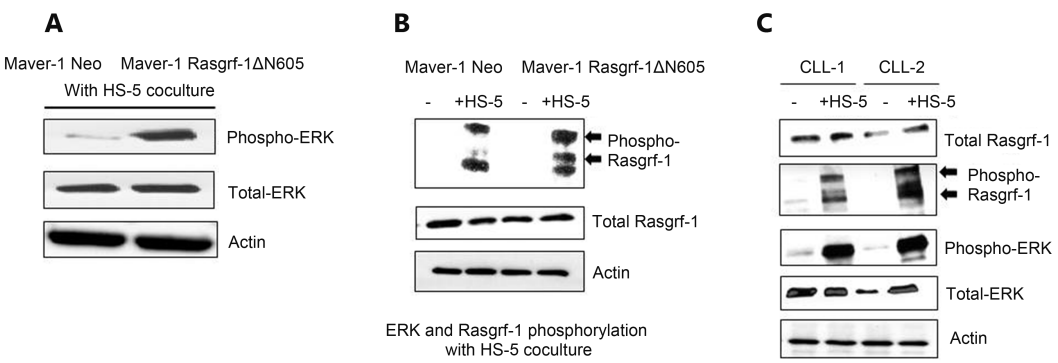

Figure 3 Modulation of Microenvironment signaling by GEF protein Rasgrf-1. (A) Maver-1 Neo and Maver-1-Rasgrf-1rN605 cells were co-cultured with HS-5 stromal cell line for 24 h. Cells in suspension were collected and lysates were analyzed for P-ERK, total ERK and actin. Maver-1-Rasgrf-1rN605cells demonstrate higher P-ERK signal with HS-5 coculture. (B) Identical lysates were also analyzed for P-Rasgrf-1, total Rasgrf-1 and actin. Western blots show that the co-culture with stromal cells results in phosphorylation of this GEF protein. In Maver-1-Rasgrf-1rN605 cells, there is phosphorylation of the additional truncated Rasgrf-1 band. (C) A similar co-culture experiment in two primary CLL specimens with lysates analyzed for total Rasgrf-1, P-Rasgrf-1, P-ERK, total ERK and actin. CLL specimens are also stimulated by stromal cell co-culture with ERK phosphorylation along with phosphorylation of the GEF, Rasgrf-1.

To determine whether the GEF plays a role in apoptosis, experiments were performed with an alkylating drug bendamustine. In the *in vivo* situation leukemic cells are in close proximity to stromal cells and are stimulated by contact, integrins and chemokines[3,4]. To replicate the *in vivo* environment, the apoptosis assays in the leukemia cells were performed with stromal cell support. Experiments were performed with Maver-1 cell lines in culture with media or in a co-culture with HS-5 cells. **Figure 4A** is a line diagram describing the percentage of apoptotic cells above background in different culture conditions. Maver-1 Neo cells are susceptible to apoptosis when treated with bendamustine, a drug often used in the treatment of MCL. When these cells are cultured with or without stromal support the percentage apoptosis is similar. However in the case of Maver-1 Rasgrf-1rN605 cells, the apoptosis in cell culture is similar to the control cells but with stromal support there is protection from apoptosis. At 5 and 10mg/mL of bendamustine, the percentage apoptosis is lower as compared to cells growing in media alone. This finding corroborates the increase in ERK phosphorylation observed in Maver-1 Rasgrf-1rN605 cells when they are co-cultured with HS-5 cells as compared to control cells (**Figure 3**). ERK pathway activation increases resistance to apoptosis and is reflected in the decrease in apoptosis when this pathway is activated by stromal cells in Rasgrf-1 over-expressing Maver-1 cells. The cell culture growth medium does not result in a differential ERK activation (**Figure 2**) and therefore the sensitivity to apoptosis is similar for control and Rasgrf-1 over-expressing cells.

Cell migration toward chemokines is required for homing, protection from apoptosis and allows leukemic cells to proliferate in a niche environment. Chemokines bind their cognate receptors that result in intracellular signaling and activation of a number of pathways including the Ras/Raf/MEK/ERK pathway. It is conceivable that over-expression of GEF could further amplify Ras signaling and this was tested in a chemotaxis assay with both the cell lines. **Figure 4B** shows the data as number of cells migrating across a membrane with and without chemokine stimulation. The Maver-1-Rasgrf-1rN605 cells are highly motile and cross the 5mm membrane even without any chemokine stimulation in a very efficient manner. There is some increase with SDF-1 chemokine stimulation as well but this increase is not significant. The Rasgrf-1 expression in Maver-1 cells alone significantly increases the ability of these cells to migrate across membranes with some additional increase in response to chemokine SDF-1. The response to SDF-1 is not significant as the Maver-1-Rasgrf-1rN605 cells are by themselves highly motile. These studies demonstrate that expression of this GEF alone is sufficient to increase the motility of these leukemic B-cells that would lead to enhanced homing and migration in tissues.

Discussion

The Ras/Raf/MEK/ERK pathway is in an important signaling pathway for B-cell malignancies including CLL and MCL. This pathway activation drives resistance to apoptosis, cell proliferation and an increase in microenvironment signaling.

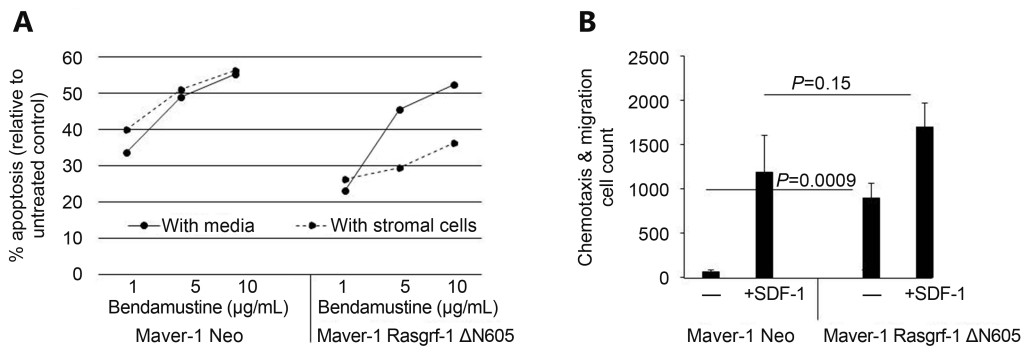

Figure 4 Resistance to apoptosis and increased chemotaxis by Rasgrf-1 over-expressing cells. (A) Maver-1 Neo and Maver-1-Rasgrf-1rN605 cells were treated with a chemotherapy drug, bendamustine, an alkylating agent. Cells were either grown in media or in co-culture with HS-5 stromal cells. 48 h after treatment cells were analyzed for apoptosis by the annexin flow cytometry assay. The line diagram represents % apoptosis (above background) at different concentrations of bendamustine. Experiment done twice with similar results. (B) Chemotaxis measured by Transwell migration of Maver-1 Neo and Maver-1-Rasgrf-1rN605 cells with and without SDF-1. SDF-1 was used at a concentration of 200 ng/mL in the bottom well and cells in the bottom well were counted by flow cytometry 4 h after adding them in the Transwell. Bar diagram shows data from three independent experiments.

Based on previous observation of high expression of this GEF protein, Rasgrf-1, in CLL and MCL we have further elucidated its mechanism of action in a MCL cell line. The studies presented here demonstrate that this GEF is acted upon by BCR and microenvironment signals and this activation upregulates Ras/Raf/MEK/ERK pathway and other BCR linked pathways.

BCR cross-linking was used a trigger to activate signaling pathways in MCL line and the results show that Maver-1 Rasgrf-1 cells are very sensitive to this signaling as lower amounts of anti-IgM antibodies were required for ERK phosphorylation as compared to control cells. In addition to phosphorylation of ERK, Rasgrf-1 is itself phosphorylated by BCR activation and it is known that this phosphorylation increases its GEF function[34]. Another explanation could be that fewer BCR cross-linked receptors are required for downstream signaling in Rasgrf-1 over-expressing cells as this GEF increases Ras-GTP levels and activates Ras/Raf/MEK/ERK and other pathways. The precise mechanism of this phosphorylation event (Ser 929) is not known but the previous studies have shown that inhibiting Src and Bruton tyrosine kinase (BTK) by specific inhibitors such as dasatinib and ibrutinib blocks Rasgrf-1 phosphorylation and ERK phosphorylation[13]. This striking result explains the role of GEF as an amplifier of BCR signaling, a critical signaling pathway for B-cell malignancies. Recent reports highlight different types of BCR interactions and low affinity BCR interactions are important for CLL pathogenesis[35]. Over-expression of this GEF will certainly enhance BCR signaling even with less than optimal BCR activation. The higher activation of Akt and PKC pathways in addition to ERK indicates a cross-talk between these pathways that activate each other. The simultaneous phosphorylation of Rasgrf-1 and ERK with BCR activation would also imply that BCR activation leads to both these signaling events.

Microenvironment, cell adhesion, and chemotaxis protect B-cell leukemias from spontaneous and drug-induced apoptosis. Residual leukemic cells residing in these protective niches after treatment are therefore potentially contributing to minimal residual disease (MRD) persistence and to disease relapse in patients after chemotherapy[3,36]. This interaction with stroma is mediated by chemokine receptors, Toll-like receptors and integrins that activate resting leukemic cells to increase their expression of CD38 and Zap-70 among other cell surface molecules. Higher activation of ERK in Rasgrf-1 expressing cells shows a similar activation that is accountable for chemoresistance and increased motility. Similar findings of chemoresistance were reported in co-culture of CLL cells with stromal cells, CD40 ligand and CpG ODN (Toll receptor agonist)[36]. Motility of cancer cells increases invasiveness and metastatic potential of cells and this is regulated by a number of pathways such as ERK, protein kinase B/Akt and Rho family of GTPases[37-39]. ERK pathway is involved in cross-linking of actin bundles that promotes cell migration by reorganization of actin cytoskeleton. Matrix metalloproteinases promote leukemic cell invasion and transendothelial migration and are activated by integrin signaling, CXCL2/CXCR4 interaction and ERK pathway activation. Furthermore MMP9 is upregulated in CLL leukemic cells, is a ERK pathway target gene [40] and will promote tissue invasion by leukemic cells.

Rasgrf-1 has a number of other domains such as pleckstrin homology domains to interact with phosphoinositides, dbl homology domain exhibits GEF activity towards Rho family of GTPases and PEST domain which is a potential target site for proteolytic cleavage[11]. Our previous results have confirmed expression of multiple truncated isoforms of Rasgrf-1 that all harbor an intact C-terminus region with the CDC25 domain that is required for GEF activity and Ras activation. In addition these truncated isoforms function as hyperactive GEFs[18,34]. An interesting observation from these studies is that even though we observe higher proliferation rate and motility of the unstimulated Rasgrf-1 over-expressing cells, the Western blot analysis does not demonstrate a higher phospho-ERK signal as compared to control cells. It plausible that there is a baseline higher phospho-ERK signal that is not discerned by the western blot analysis or Rasgrf-1 has additional functions besides activating Ras.

The evidence highlights a role of Rasgrf-1 similar to Zap-70, a tyrosine kinase in CLL which is a well-known prognostic marker of aggressive disease. Zap-70 expression is linked to CLL leukemic cells with a higher proliferative, migratory potential and its expression is upregulated by stromal co-culture, CD40ligand and Toll receptor agonists[36,41,42]. Rasgrf-1 functions in a similar way in the MCL model as there is higher ERK activation with stromal co-culture in Rasgrf-1 over-expressing cells. Zap-70 also increases BCR signaling as there is increase in tyrosine phosphorylation of Syk, ERK and Akt in cells which have a higher Zap-70 expression[43-45]. Similar results were obtained with high Rasgrf-1 expressing cells with stronger activation of a number of BCR downstream effector pathways such as ERK, PKC and Akt.

Of all the B-cell malignancies, Rasgrf-1 over-expression is limited to CLL and MCL cells[13]. The experiments with ectopic over-expression of transgenes are not technically feasible in the CLL model as these primary cells are not easily transfectable. The Maver-1 line is activated both by BCR cross-linking and with co-culture with stromal cells, similar

to CLL leukemic cells. In addition there is an overlap in therapeutic agents that are active in these two malignancies. There are however caveats as MCL cells harbors unique genetic alterations that are not observed in CLL such as cyclin D1 translocation t(11:14). In our previous study, we performed SiRNA based inactivation of Rasgrf-1 in CLL specimens and observed decrease in Ras-GTP and inhibition of ERK phosphorylation with BCR activation in leukemic cells that were transfected with Rasgrf-1 SiRNA. Findings in this study with MCL lines corroborate previous results as over-expression of Rasgrf-1 clearly results in higher ERK phosphorylation by BCR and microenvironment signals.

Overall our studies show that Rasgrf-1 over-expression increases Ras/Raf/MEK/ERK pathway signaling in B-cell malignancies. This pathway is activated by a number of stimuli and Rasgrf-1 further augments these signals by increasing Ras activation. The downstream effectors of this pathway promote apoptotic resistance, motility and chemokine signaling and affect biology of B-cell malignancies. Rasgrf-1 is phosphorylated by BCR and stromal growth factors that strengthen the argument that this GEF has a central role in the pathogenesis of B-cell malignancies. This over-expression is not only upregulating the ERK pathway but a number of other well studied critical CLL pathways such as Akt and PKC by cross-talk mechanism. Blocking its function and interaction with Ras will conceivably inhibit Ras signaling and will be therapeutic strategy for B-cell malignancies.

Conflict of interest statement

No potential conflicts of interest are disclosed.

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