Clinical & Experimental Metastasis **19:** 301–311, 2002.

Mitogen activated protein kinase pathway is involved in RhoC GTPase induced motility, invasion and angiogenesis in inflammatory breast cancer

Kenneth L. van Golen[1], Li Wei Bao[1], Quintin Pan[1], Fred R. Miller[2], Zhi Fen Wu[1] & Sofia D. Merajver[1]
[1]*Department of Internal Medicine, University of Michigan Comprehensive Cancer Center, Ann Arbor, Michigan, USA;* [2]*Breast Cancer Program, Barbara Ann Karmanos Cancer Institute, Detroit, Michigan, USA*

Received 24 August 2001; accepted in revised form 4 December 2001

Key words: angiogenic factors, C3 exotransferase, human mammary epithelial (HME) cells, inflammatory breast cancer (IBC), inhibitors, invasion, mitogen activated protein kinase (MAPK), motility, phosphatidylinositol-3 kinase (PI3K), RhoC GTPase

Abstract

Inflammatory breast cancer (IBC) is the most lethal form of locally advanced breast cancer known. IBC carries a guarded prognosis primarily due to rapid onset of disease, typically within six months, and the propensity of tumor emboli to invade the dermal lymphatics and spread systemically. Although the clinical manifestations of IBC have been well documented, until recently little was known about the genetic mechanisms underlying the disease. In a comprehensive study aimed at identifying the molecular mechanisms responsible for the unique IBC phenotype, our laboratory identified overexpression of RhoC GTPase in over 90% of IBC tumors in contrast to 36% of stage-matched non-IBC tumors. We also demonstrated that overexpression of RhoC GTPase in human mammary epithelial (HME) cells nearly recapitulated the IBC phenotype with regards to invasion, motility and angiogenesis. In the current study we sought to delineate which signaling pathways were responsible for each aspect of the IBC phenotype. Using well-established inhibitors to the mitogen activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K) pathways. We found that activation of the MAPK pathway was responsible for motility, invasion and production of angiogenic factors. In contrast, growth under anchorage independent conditions was dependent on the PI3K pathway.

Abbreviations: ELISA – enzyme linked immunoabsorbant assay; FBS – fetal bovine serum; FGF2 – basic fibroblast growth factor; FGF-BP – fibroblast growth factor binding protein; HME – human mammary epithelial; IBC – inflammatory breast cancer; IGFBP-rP – insulin-like growth factor binding protein related protein; IL – interleukin, LABC – locally advanced breast cancer; MAPK – mitogen activated protein kinase; MEM – minimal essential medium; MTT – 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PI3K – phosphatidylinositol-3 kinase; TCA – tricholoracetic acid; VEGF – vascular endothelial growth factor

Introduction

Inflammatory breast cancer (IBC) is a phenotypically distinct form of locally advanced breast cancer (LABC) that has a propensity to invade, grow and spread in the dermal lymphatics of the skin overlying the breast [1–3]. It is the ability of the tumor emboli to invade and block the dermal lymphatics that leads to its poor prognosis [1–4].

Although the clinical manifestations of IBC have been well documented in the literature, until recently little was known about the molecular mechanisms involved in conferring the unique IBC phenotype. In an effort to identify genetic alterations involved in determining the IBC phenotype, our laboratory isolated two genes that were consistently and concordantly altered in IBC compared with stage-matched non-IBC tumors [5]. RhoC GTPase, was found to be over-expressed in 90% of IBC tumors versus 36% of the stage-matched controls.

RhoC GTPase is a member of the Ras-superfamily of small GTP binding proteins and is primarily responsible for re-organization of the actin cytoskeleton leading to the formation of lamellipodia and fillipodia resulting in cellular motility [6–13]. Transfection of the RhoC homologue, RhoB, into Ras-transformed NIH3T3 cells leads to increased focus formation suggesting a role for the Rho proteins as a transforming oncogene or as a metastasis gene [14]. Similarly, our laboratory has demonstrated that RhoC transfected HME cells become highly motile and invasive, grow under anchorage independent conditions, produce angiogenic factors, and are tumorigenic and metastatic when orthotopically implanted into nude mice [15–17].

Correspondence to: Sofia D. Merajver, MD, PhD, Department of Internal Medicine, University of Michigan Comprehensive Cancer Center, 7217 CCGC, 1500 East Medical Center Drive, Ann Arbor, MI 48109-0948, USA. Tel: +1-734-764-2248; Fax: +1-734-615-2719; E-mail: smerajve@umich.edu

These *in vitro* data have additional clinical significance as RhoC overexpression is associated with the transition to metastatic disease in other cancers [18–20]. It has been proposed that Rho proteins act through and potentiate signaling via the c-Jun kinase/stress activated protein kinase (JNK/SAPK) and mitogen activated protein kinase (MAPK) pathway (reviewed by Takai et al. [21]). Evidence from other laboratories suggest that Rho proteins can signal through both the MAPK pathway as well as the phosphoinositol-3 kinase (PI3K) pathway, while cdc42 and Rac1 are associated with the JNK/SAPK pathway [22–25]. Furthermore, it has been demonstrated that in certain cell types, the MAPK pathway is involved in signaling and the production of angiogenic factors while the PI3K pathway is involved in growth and survival [26–33].

In the present study we set out to determine the major pathways involved in RhoC signaling in IBC. Specifically, we attempted to determine which pathways and cascades were involved in conferring specific aspects of the RhoC-induced phenotype. Many of the published studies that describe the signal transduction pathways involved in Rho signaling were performed in transfected NIH3T3 cells, thus our study focused on the RhoC signaling pathways specific to IBC and HME cells. We treated HME-RhoC stable transfectants, control HME-β-galactosidase (HME-β-gal) transfectants or the SUM149 IBC cell line with C3 exotransferase (a specific inhibitor of Rho proteins), a variety of MAPK inhibitors, or a PI3K inhibitor and assayed them for specific biological functions. The inhibitors were used at concentrations that would inhibit signal transduction without affecting cellular viablility. We found that the PI3K pathway was involved in anchorage independent growth and survival, while multiple arms of the MAPK pathway were involved in motility and invasion, and that p38 is a downstream modulator in the production of angiogenic factors. These data provide significant new insight as to how overexpression of RhoC can lead to a variety of phenotypic effects in breast cells.

Materials and methods

Cell culture

Cell lines were maintained under defined culture conditions for optimal growth in each case [34–36]. Briefly, human mammary epithelial (HME) cells were immortalized with human papilloma virus E6/E7 [37] and grown in 5% fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, Missouri) supplemented Ham's F-12 medium (JRH BioSciences, Lenexa, Kansas) containing insulin, hydrocortisone, epidermal growth factor, and cholera toxin (Sigma Chemical Co.). Stable HME transfectants containing either the human wild-type RhoC GTPase or control β-galactosidase genes were produced and maintained in the described medium supplemented with 100 μg/ml hygromycin (LifeScience Technologies) as previously published [15–17]. The SUM149 cell line was developed from a primary IBC tumor and grown in 5% FBS supplemented Ham's F-12 medium containing insulin and hydrocortizone. The HME cells were characterized as being keratin 19 positive, ensuring that they are from the same differentiation lineage as the SUM149 IBC tumor cell line [5].

Cells actively growing in culture were treated with MAPK inhibitors, 2.0 μM PD98059, 1.5 μM U0126, 1.5 μM SKF86002, or 1.5 μM SB220025 (all obtained from Calbiochem, San Diego, California) 24 h prior to assays and treated everyday with fresh inhibitor until the end of the assay. Treatment of cells with 2.5 μM LY294002 (Calbiochem), a PI3K inhibitor, was performed in the same manner as described for the MAPK inhibitors. These concentrations were below the IC_{50} of the compounds to avoid direct cell toxicity to allow for meaningful biological assays.

Western blot analysis

Proteins were harvested from cell cultures using RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml PMSF, 1 mM sodium orthovanadate and 0.3 mg/ml aprotinin; Sigma Chemical Co.). Ten μg aliquots were mixed with Laemelli buffer, heat denatured for 3 min, separated by SDS-PAGE, and transferred to nitrocellulose. Non-specific binding was blocked by overnight incubation with 2% powdered milk in tris-buffered saline with 0.05% Tween-20 (Sigma Chemical Co.). Immobilized proteins were probed using antibodies specific for total MAPK proteins, the membranes were then stripped and reprobed for the phosphorylated form of the MAPK protein. Specifically, p38/pp38, pJNK/ppJNK, and pErk/ppErk (Cell Signaling Technologies, Beverly, Massachusettes). Protein bands were visualized by ECL (Amersham-Pharmacia Biotech, Piscataway, New Jersey).

C3 exotransferase treatment

Active C3 exoenzyme was introduced into the HME, HME-β-gal, HME-RhoC, and SUM149 cells using a method based on liposome encapsulation and membrane fusion, which we have termed lipoporation [16]. Briefly, cells were grown in 6-well plates until reaching a confluence of 40–50% and the medium replaced with fresh medium. Three micrograms of human recombinant C3 exotransferase (Cytoskeleton Inc., Denver, Colorado) was combine with FuGene^{TM} 6 transfection reagent (Roche-Boehringer Mannheim) and added to the cultures. As controls either an equal quantity of human recombinant tubulin or FuGene^{TM} 6 alone were added to cell cultures. The cells were incubated for 2 days at 37 °C, at which time cell-conditioned medium was harvested. Presence of the intracellular C3 exoenzyme was confirmed by visualizing the rhodamine-tagged protein using fluorescent microscopy. The efficiency and activity of both the transfected and lipoporated C3 exoenzyme were confirmed by a quantitative ADP-ribosylation assay [38].

The efficiency of *in vivo* ADP-ribosylation of RhoC GTPase by C3 exotransferase was determined as previously described [16]. Active C3 exotransferase was efficiently introduced into HME-β-gal, HME-RhoC, and SUM149, as described above. Cells were collected 48 h later, washed

in medium, and pelleted. The cells were lysed in 20 mM HEPES pH 8.0 (Sigma Chemical Co.) by 3 repeated freeze/thaw cycles. Cell lysates (10 μg) were combined with 50 ng/ml C3 exotransferase and 5×10^6 cpm (with a specific activity of 1×10^6 cpm/μl) [^{32}P]NAD (Amersham) in ADP-ribosylation buffer (20 mM HEPES, pH 8.0, 1 mM $MgCl_2$, 1 mM AMP and thymadine, Sigma Chemical Co.), and incubated for 30 min at 37 °C. TCA-percipitable material was then recovered and radioactivity was counted on a Packard scintillation counter.

Growth assays

Monolayer culture growth rate was determined as previously described [39] by conversion of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma Chemical Co.) to a water insoluble formazon by viable cells. Three thousand cells in 200 μl medium were plated in 96-well plates and grown under normal conditions. Cultures were assayed at 0, 1, 2, 3, 5 and 7 days by the addition of 40 μl 5 mg/ml MTT and incubating for 1 h at 37 °C. The time points of the assay were chosen to sufficiently discern any effect that the inhibitors may have on cell viability, which may affect the outcomes of the phenotypic experiments. The MTT containing medium was aspirated and 100 μl DMSO (Sigma Chemical Co.) added to lyse the cells and solublize the formazon. Absorbance values of the lysates were determined on a Dynatech MR 5000 microplate reader at 540 nm.

For anchorage independent growth assays, a 2% stock of sterile low-melt agarose was diluted 1:1 with 2× MEM. Further dilution to 0.6% agarose was made using 10% FBS supplemented Ham's F-12 medium complete with growth factors, and 1 ml was added to each well of a six-well plate as a base-layer. The cell layer was then prepared by diluting agarose to 0.3% and 0.6% with 10^3 cells in 2.5% FBS supplemented Ham's F-12/1.5 ml/well. Colonies greater than or equal to 100 μ in diameter were counted after a 3-week incubation at 37 °C in a 10% CO_2 incubator.

Random motility assay

Random motility was determined using a gold-colloid assay [40]. Gold-colloid (Sigma Chemical Co.) was layered onto glass coverslips and placed into 6-well plates. Cells were plated onto the coverslips and allowed to adhere for 1 h at 37 °C in a CO_2 incubator (12 500 cells/3 ml in serum-free medium). To stimulate the cells, the serum-free medium was replaced with 5% FBS containing Ham's F-12 supplemented with growth factors and allowed to incubate for 3 h at 37 °C. The medium was aspirated and the cells fixed using 2% gluteraldehyde (Sigma Chemical Co.). The coverslips were then mounted onto glass microscope slides and areas of clearing in the gold-colloid corresponding to phagokinetic cell tracks counted.

Invasion assay

The invasion assay was performed as previously described with minor modification [39]. A 10 μl aliquot of 10 mg/ml Matrigel (Becton Dickenson, Bedford, Massachusetts) was spread onto a 6.5 mm Transwell filter with 8 μm pores (Costar, Corning, New York) and air dried in a laminar flow hood. Once dried, the filters were reconstituted with a few drops of serum-free medium. The lower chamber of the Transwell was filled with either serum-free or serum containing media. Cells were harvested and resuspended in serum-free medium with 0.1% BSA at a concentration of 3.75×10^5 cells/ml and 0.5 ml was added to the top chamber. The chambers were incubated for 24 h at 37 °C in a 10% CO_2 incubator. The cell suspension was aspirated and excess Matrigel removed from the filter using a cotton swab. The filters were then cut away from the Transwell assembly and fixed with methanol, gel side down, to a glass microscope slide. The fixed filters were stained with hematoxylin and eosin, and the cells on the entire filter were counted at a 40×-magnification individually by two investiagtors. These cells were assumed to have invaded through the Matrigel and filter. The number of cells that had invaded in the serum-free containing lower chambers was considered background and this number was subtracted from the number of cells that had invaded in response to the serum-containing medium.

Quantitation of vascular endothelial growth factor

Levels of soluble cytokines and chemokines were determined from cell-conditioned media. Cells were incubated in normal growth medium for four days. The cell-conditioned media was harvested, centrifuged for 5 min at 2,500 rpm and divided into 1 ml aliquots. The Quantikine human vascular endothelial growth factor (hVEGF; R&D Systems, Minneapolis, Minnesota) were used to measure protein levels of the 165 amino acid species of hVEGF. The enzyme linked immunoabsorbant assay (ELISA) was performed per the manufacturers recommendations.

Results

C3 exotransferase inhibition of RhoC GTPase

In a previous study we demonstrated that inhibition of RhoC GTPase activity by C3 exotransferase treatment led to decreased production of angiogenic factors [16, 17]. In order to demonstrate that the other phenotypic changes seen in the HME-RhoC transfectants are indeed due to RhoC expression, we treated the cells with C3 exotransferase. C3 exotransferase is not a specific inhibitor of RhoC *per se*, but a specific inhibitor of Rho proteins (reviewed in [41]). C3 has been demonstrated to have an affinity for RhoC and affects the formation of actin filaments *in vivo* [42]. Given that the untransfected HME, the HME-β-galactosidase control transfectants, and the HME-RhoC transfected cells were all derived from the same culture, they are likely to share the same distribution of Rho proteins, except for RhoC. Therefore, main changes of phenotype produced by C3 treatment would be ascribed to C3 induced changes in RhoC GTPase activity.

Table 1. Comparison of monolayer population doubling time and anchorage independent growth of untreated and C3 treated HME, HME transfectants and SUM149 IBC cell lines. Despite treatment of the cells with C3, monolayer population doubling time was not affected. In contrast, the ability of the RhoC expressing HME and SUM149 cells to grow under anchorage independent conditions was significantly reduced ($^{*}P = 0.01$, $^{**}P = 0.001$).

	Population doubling time (hours)		Anchorage independent growth (number of cholonies)	
	Untreated	C3 Treated	Untreated	C3 Treated
HME	34 h	36 h	0 ± 0	0 ± 0.1
HME-β-gal	35 h	34 h	5 ± 0.8	17 ± 7.5
HME-RhoC	33 h	36 h	102 ± 5.4	$40 \pm 13.6^{**}$
SUM149	39 h	39 h	75 ± 4.9	$47 \pm 3.3^{*}$

Active C3 exotransferase was introduced into the cells using a liposome mediated method termed lipoporation [16]. As shown in Table 1, the population doubling time of all the cell lines tested was not significantly affected by C3 treatment. However, the ability of the HME-RhoC transfectants and the SUM149 IBC cell line to grow under anchorage independent conditions, a hallmark of malignant transformation, was significantly reduced. In contrast, C3 treatment of the HME untransfected or the HME-β-gal control did not result in any changes in their ability to grow in soft agar. The monolayer growth rate was not influenced by transfection or RhoC expression, as the HME-RhoC transfectants did not differ from the untransfected or control transfected HME counterparts, or by C3 treatment so, these data suggest that RhoC confers the ability to HME-RhoC cells to grow under anchorage independent conditions.

As demonstrated in Figure 1A, C3 treatment significantly reduced HME-RhoC and SUM149 IBC motility in a random colloidal gold assay. The HME-β-galactosidase control transfectants were unaffected by C3 treatment. Similarly, the ability of the HME-RhoC and SUM149 cells to invade a Matrigel coated filter in response to a chemoattractant was significantly reduced after C3 treatment (Figure 1B).

The activity of the C3 exotransferase was confirmed by measuring the efficiency of *in vivo* ADP-ribosylation. As shown in Figure 1C, in comparison with their non-C3 treated counterparts, all the C3 treated cell lines had a significant reduction in the levels of available sites that could be ADP-ribosylated in the *in vitro* assay. Specifically, the C3-treated HME-RhoC and SUM149 cells had a 2-fold decrease in the number of ADP-ribosylated sites compared to the non-transfected controls. These data indicate that at least half of the Rho proteins have been ADP-ribosylated *in vivo*, and therefore inhibited by C3 exotransferase. Taken together, these data demonstrate that expression and activity of RhoC GTPase is responsible for conferring the ability to grow under anchorage independent conditions, and the production of a motile and invasive cell.

Inhibition of anchorage independent growth by the LY294002 PI3K inhibitor

To determine whether the PI3K or the MAPK pathways were involved in RhoC signaling, the cells were treated with either LY294002 (a potent PI3K inhibitor) or PD98059 (a general MAPK inhibitor that blocks all arms of the MAPK pathway). To avoid confounding effects due to direct cytotoxicity, we chose concentrations of the inhibitors that inhibited signal transduction but were not cytotoxic. The cells were treated 48 h prior to plating in 0.6% soft agar and fresh medium containing each of the inhibitors was layered onto the soft agar daily. The MCF10AT c1 cell line, with a constituitvely active Ras was used as a positive control [43]. The ability of the HME-RhoC and SUM149 cells to form colonies in 0.6% soft agar was significantly reduced by treatment with the PI3K inhibitor (Figure 2). In contrast, treatment with the general MAPK inhibitor PD98059 had little effect on the colony number. The reduction in colony formation was not due to a significant change in the population doubling time of the cells treated with LY294002, as determined by an MTT monolayer growth assay performed on cells treated long-term with the inhibitors (data not shown). These data indicate the PI3K pathway, and not the MAPK pathway is involved in RhoC conferring the ability of the cells to survive and form colonies under anchorage independent conditions.

MAPK status in cell lines after inhibitor treatment

In order to determine which arms of the MAPK pathway were involved in the different aspects of the RhoC-induced phenotype, the cells were treated with a variety of MAPK inhibitors that affect different points of the pathway. The general MAPK inhibitor PD98059 effects the MAPK pathway at 2 distinct, points; (1) MEKK-1 (which activates p38 and MEK1 & 2, and therefore ERK1 & 2), and (2) directly at MEK1 & 2. The inhibitor U0126 specifically inhibits MEK1 & 2 activation. The inhibitors SKF86002 and SB22025 are inhibitors of p38 activation and of p38 itself, respectively. As demonstrated in Figure 3, all cell lines expressed p38, ERK (p42/p44), and JNK/SAPK. However, none of the untreated cell lines (A) expressed activated phospho-JNK/SAPK, sug-

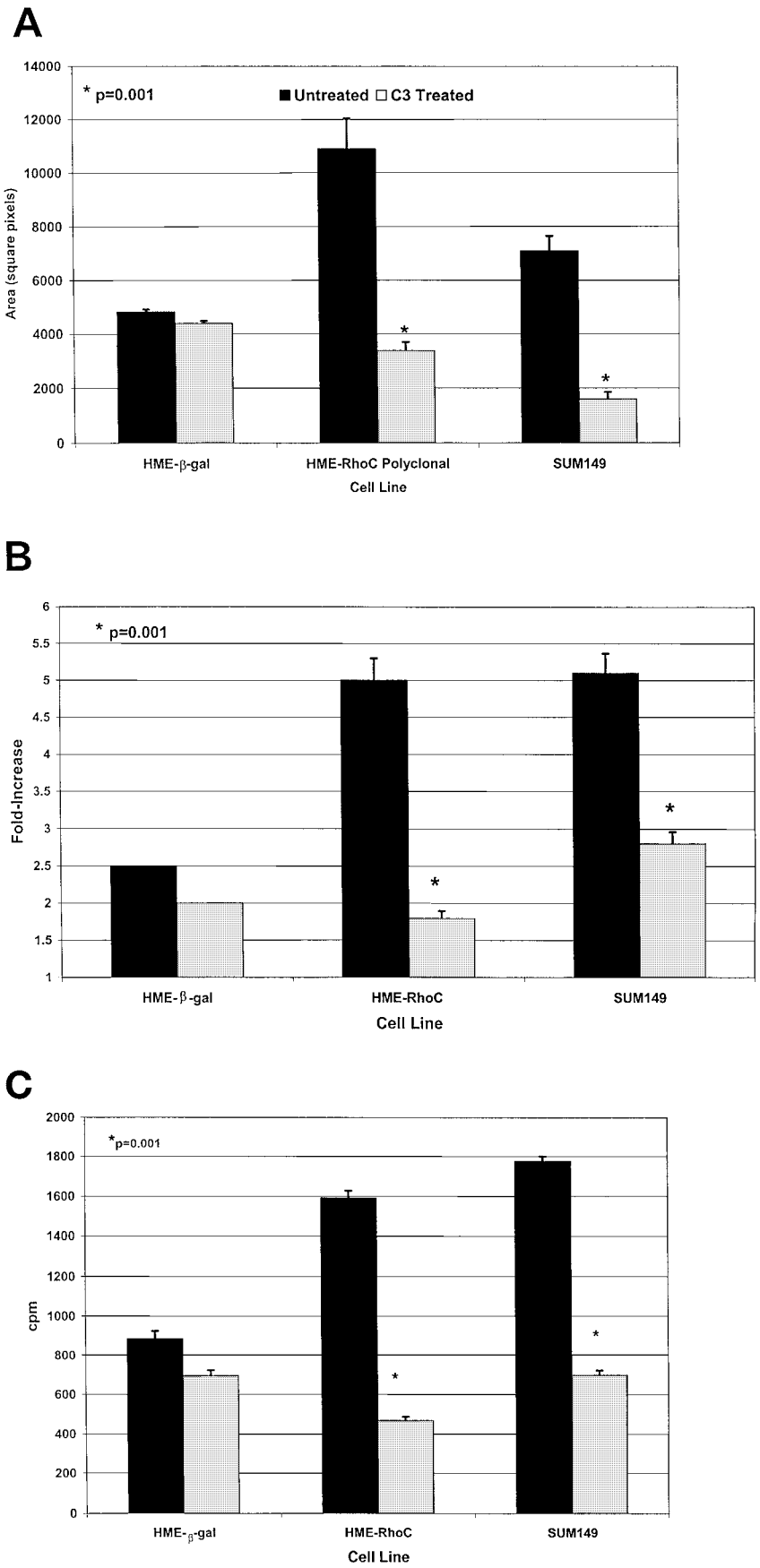

Figure 1. Comparison of the average area of migration in a colloidal gold motility assay by control HME-β-gal and RhoC overexpressing mammary cells after treatment with C3 exotransferase. Treatment of cells with C3 exoenzyme significantly reduced the motile ability of the RhoC overexpressing cells HME-RhoC and SUM149. Similarly, as demonstrated in panel B, the invasive capabilities of the RhoC overexpressing cells were also significantly reduced after C3 treatment as determined in a Matrigel invasion assay. To determine the extent of Rho inhibition by C3 exotransferase, an *in vitro* ADP-ribosylation assay was performed (panel C). The number of ADP-ribosylated targets was greatly reduced in the HME-RhoC and SUM149 cells, thus indicating that C3 exotransferase treatment had effectively blocked the Rho targets within those cells.

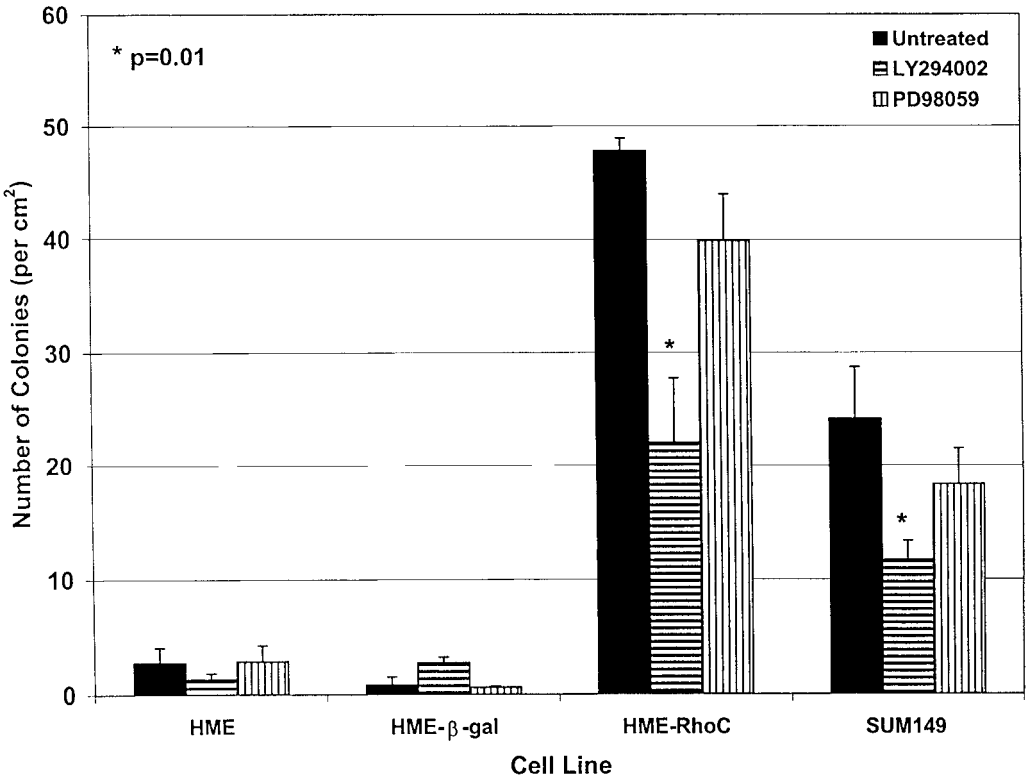

Figure 2. Anchorage independent growth in 0.6% soft agar after treatment with either the PI3K inhibitor LY294002 or the general MAPK inhibitor PD98059. The ability of the RhoC overexpressing cells HME-RhoC and SUM149 was significantly reduced after treatment with the LY294002, but not with PD98059. These data suggests that RhoC-mediated anchorage independent growth, is signaled through the PI3K and not the MAPK pathway in these mammary cells.

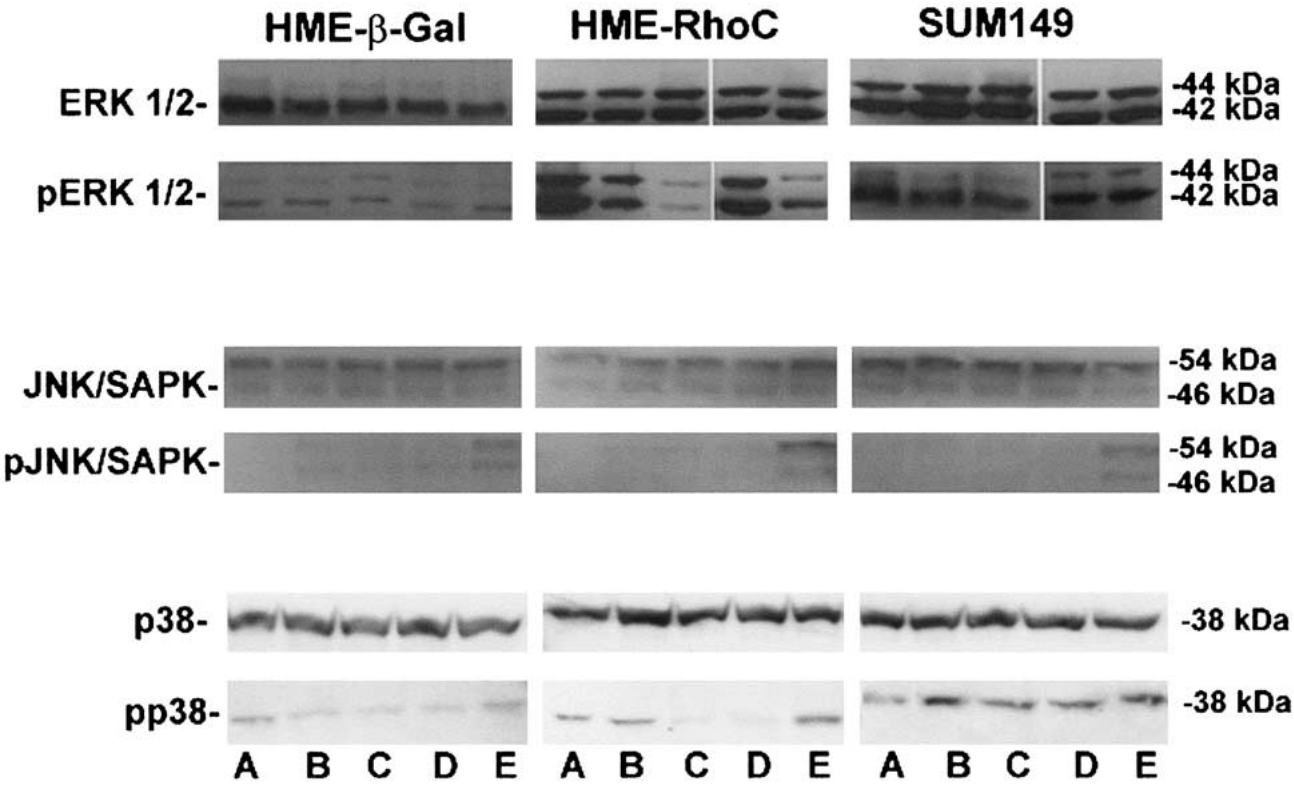

Figure 3. Western blot analysis of basal and phosphorylated (activated) levels of different arms of the MAPK pathway before (A) and after inhibitor treatment with SKF86002 (B), PD98059 (C), U0126 (D), or C3 exotransferase (E). All the cell lines tested expressed ERK (p42/p44), JNK/SAPK, and p38. However, none of the untreated cell lines (A) expressed activated phospho-JNK/SAPK, suggesting that only pp38 and phospho-ERK are involved in RhoC signal transduction. Each set of Western blots looking at p38, JNK/SAPK and ERK were performed separately on the same cell lysates. Each individual blot in the set (i.e. total protein *versus* the phosphorylated form of that protein) was the same blot stripped and re-probed.

gesting that only pp38 and phospho-ERK are involved in RhoC signal transduction. Each set of Western blots looking at p38, JNK/SAPK and ERK were performed separately on the same cell lysates. Each individual blot in the set (i.e., total protein versus the phosphorylated form of that protein) was the same blot stripped and re-probed. Each set of Western blots looking at p38, JNK/SAPK and ERK were performed separately on the same cell lysates. Each set of Western blots compares total protein *versus* the phosphorylated form of that protein of the same blot. Treatment of the cells for 24 h with SKF86002 (B), PD98059 (C), U0126 (D), or C3 exotransferase (E), led to a decrease in the levels of the active phosphorylated form of the target protein(s), while the basal levels remained unchanged. Interestingly, C3 treatment of the cells lead to increased phosphorylation of JNK/SAPK.

Effect of inhibition of MAPK on motility and invasion

Because of the postulated relationship between Rho-induced motility and Ras activation of the MAPK pathway, we set out to understand how are the MAPK signaling cascades are involved in Rho-modulated motility and invasion. To accomplish this we treated the cells with the various MAPK inhibitors described above. The cells were treated with the MAPK inhibitors 48 h prior to assessing motility and invasion. No significant decrease in population doubling time was observed for the cells treated with inhibitors, as determined over a seven-day assay (data not shown).

As demonstrated in Figure 4A, all of the MAPK inhibitors had a significant ($P = 0.01$) effect on the motility of the HME-RhoC and SUM149 cell lines. The areas of the phagokinetic tracks were reduced to nearly the level of the HME-β-gal control cell line, which was unaffected by any of the MAPK inhibitors. Since all of the MAPK inhibitors had an effect on the motility of the cells, this suggested that multiple arms of the MAPK pathway are involved in RhoC mediated motility. Motility of the MCF10AT c1 positive control cell line that has a constituatively active Ras was also affected by all four of the MAPK inhibitors, although the motility of these cells is much reduced compared to the HME-RhoC and SUM149 cells.

Next, we concentrated on the cells ability to invade through a Matrigel coated filter (Figure 4B). The invasive capabilities of the cells are described as fold-increase in invasion over untransfected HME controls. Treatment with all four of the MAPK inhibitors reduced the invasive capabilities of the HME-RhoC and SUM149 cell lines. The HME-β-gal control cells were not significantly affected, by the other MAPK inhibitors. The invasive capabilities of the MCF10AT c1 cells were the same as the HME-β-gal control cells, and were likewise unaffected by the MAPK inhibitors. When cells were treated with a combination of the LY294002 and PD98059 inhibitors, the level of inhibition was similar to that of the PD98059 inhibitor alone (data not shown), suggesting that the PI3K pathway is not involved in either motility or invasion.

Taken together, these data suggest that RhoC induced motility and invasion is mediated to a significant extent by the p38 and ERK arms of the MAPK pathway. This is shared with Ras alone induced motility, but active Ras is not sufficient to produce an invasive phenotype in the MCF10A cells. In all these experiments the concentrations of inhibitors used did not effect cell doubling times or cell viability.

VEGF production after inhibition of the MAPK pathway

In a previous study, we demonstrated that RhoC overexpression leads to increased production of angiogenic factors, particularly vascular endothelial growth factor (VEGF) [16, 17]. Since VEGF production was significantly increased in RhoC expressing breast cells, and considering its importance in as an angiogenic factor, VEGF expression was the logical endpoint to study after inhibitor treatment. Treatment of the mammary cells with the different MAPK inhibitors resulted in decreased VEGF production by the HME-RhoC and SUM149 IBC cell lines (Figure 5). The greatest reduction in VEGF production was seen when the cells were treated with the inhibitor SB22025, which prevents p38 activation. Treatment with the inhibitor SKF86002, an inhibitor of phosho-p38 activity, resulted in the second greatest decrease in VEGF production. Taken together, these data suggest that activation of the p38 arm of the MAPK pathway is responsible for production of VEGF simultaneously by RhoC overexpression.

Discussion

The highly invasive and metastatic phenotype of IBC is one of the hallmarks of its unique clinical manifestations and the major cause of the poor outcome of many patients who are diagnosed with IBC. In a previous set of studies, our laboratory identified RhoC GTPase to be overexpressed in IBC and responsible for anchorage independent growth, cellular motility and invasion, and production of angiogenic factors.

In the current study, we begin to delineate the signaling pathways responsible for each aspect of the RhoC-mediated phenotype. We hypothesized that for RhoC GTPase to achieve diverse phenotypic attributes, cell signaling must take place through several signal transduction pathways. We utilized specific inhibitors of different points of the PI3K and MAPK pathways, an approach which has proven successful in similar previous studies [44, 45].

The MAPK pathway has been previously implicated in Ras and RhoA signaling, while the JNK/SAPK pathway has been shown to be mediated by Rac1 and cdc42 signaling, and all have been shown to use the PI3K pathway (reviewed in [46, 47]). These pathways, depending on cell type, have been attributed to participate in growth/survival and motility (reviewed in [29]). However, the signal transduction pathway(s) utilized by RhoC during growth, motility and invasion has not been described in any cell type. In the current study we examined RhoC signal transduction in the SUM149 IBC and HME-RhoC breast cell lines. Because RhoC appears to be a major determinant of a clinically well-defined mammary cancer metastatic phenotype it is es-

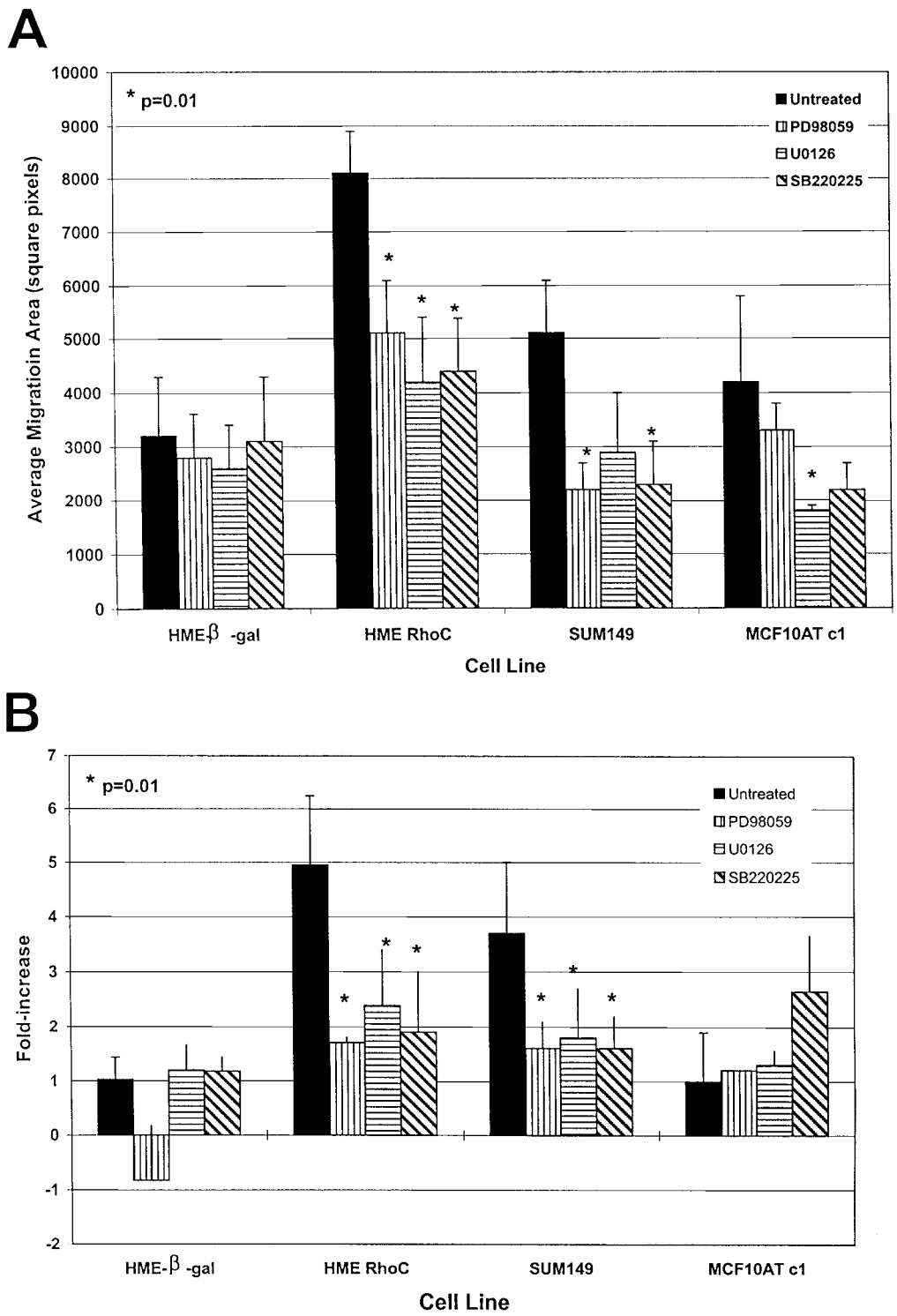

Figure 4. Effects on motility and invasion of RhoC overexpressing cells after treatment with PD98059, U0126 or the p38 inhibitor SB220225. Panel A demonstrates a significant decrease in the motility of the RhoC overexpressing HME-RhoC and SUM149 cells treated with the various MAPK inhibitors. Similarly, the ability of these cells to invade through a Matrigel coated filter was also significantly reduced after treatment with the MAPK inhibitors (panel B). These data suggest that RhoC mediated motility and invasion is mediated through the MAPK pathway, to a large extent through activated p38.

pecially relevant to understand how RhoC elicits multiple actions in breast tissue.

Using MAPK and PI3K inhibitors at concentrations below cytotoxic and cytostatic levels, we have determined that the PI3K pathway is involved in the ability of RhoC overexpressing cells to grow under anchorage independent conditions without effecting monolayer growth. We also determined that signaling through the MAPK pathway is involved in motility, invasion and the production of angiogenic factors. Specifically, we found that the ERK and p38 arms of the MAPK signaling complex are involved in motility and invasion, as no one inhibitor of the individual arms com-

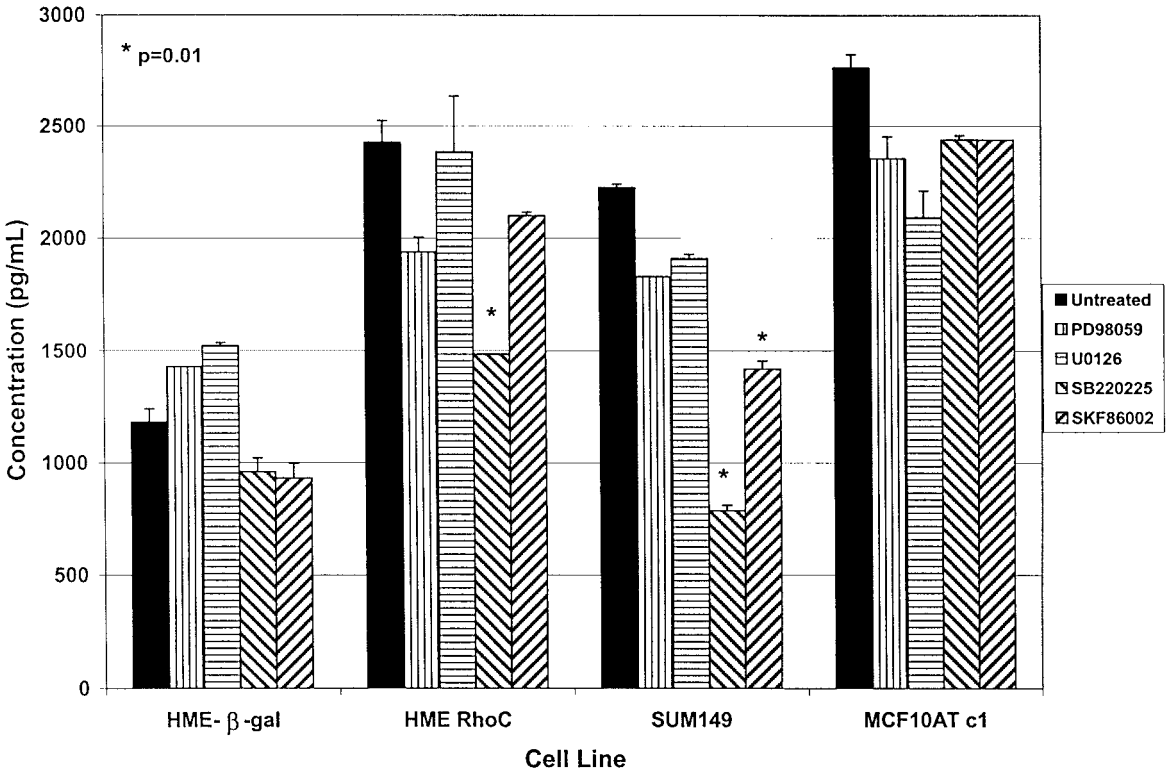

Figure 5. The effect of MAPK inhibitors on the production and secretion of the pro-angiogenic molecule vascular endothelial growth factor (VEGF). Production of VEGF by the HME-RhoC and SUM149 cell lines were significantly reduced when the cells were treated with the p38 inhibitors SB220225 or SKF86002. Taken together, these data suggest that activation of the p38 arm of the MAPK pathway is responsible for production of VEGF simultaneously by RhoC overexpression.

pletely blocked motility of the HME-RhoC or SUM149 IBC cell lines. In addition, although it is expressed, we know that JNK/SAPK does not appear to be involved given that it is not phosphorylated or active in any of the breast lines (IBC or HME) studied.

Previous experiments have demonstrated that the various signal transduction pathways have diverse effects in different cell types activated by a variety of stimuli. For example in Schwann cells, PI3K activation by Rac1 leading to lamellipodia formation and motility has been shown to occur upon stimulation by insulin-like growth factor-I [25]. Whereas stimulation of adipocytes with insulin leads activation of the PI3K pathway and Rho-mediated glucose uptake [48]. In support of our data, Amundadottir and Leder [49] demonstrated that regardless of the oncogene involved in transformation, the PI3K pathway was involved in conferring anchorage independent growth to transformed mammary epithelial cells. They also demonstrated that anchorage independent growth of mammary cells transformed by Her2/neu, v-Ha-ras, and c-myc, could not be inhibited by treatment with the MAPK inhibitor PD98059. Thus consistent with our study of RhoC-expressing cells, it appears that the PI3K pathway is exclusively involved in conferring anchorage independent growth, without involving the MAPK pathway.

Several studies have demonstrated that activation of the MAPK pathway can lead to cell migration and invasion of fibroblasts, keratinocytes and endothelial cells [50–52]. Further, it has been well documented that the Rho proteins can activate the MAPK cascade stimulating various aspects of cellular motility [29, 53–55]. Rac1 and cdc42 have been shown to signal gene transcription through JNK/SAPK and RhoA through p38, or when bound to fibronectin, ERK 1 & 2 [22, 53, 56, 57]. Akin to RhoA, we have made similar observations for RhoC, having demonstrated activation of both p38 and ERK, but not JNK/SAPK, in IBC and transfected HME cells. During motility, a dynamic interplay between Rac1, cdc42 and Rho must occur to form lamellipodia, fillipodia, focal adhesions, and stress fibers [7, 58, 59]. 'Cross-talk' between these molecules results in reciprocal activation of Rho with Rac1 and cdc42 [59–61]. Therefore, each arm of the MAPK pathway may be involved in motility and invasion during some point of the process.

In a previous study we demonstrated that levels of VEGF was significantly elevated due to RhoC overexpression [16]. In support, other laboratories have demonstrated that expression of angiogenic factors is mediated by the p38 MAPK cascade [26–28]. An increasing body of evidence suggests that the mode of VEGF induction (i.e., MAPK vs. PI3K) by activated H-*ras* is a cell-type specific process, with cells of epithelial origin signaling more commonly through the MAPK pathway and those cells of mesodermal origin utilizing the PI3K pathway [62]. Stimulation of a variety of breast cancer cell lines with heregulin results in activation of p38 and subsequent upregulation of VEGF expression and secretion [28]. Similarly, epidermal growth factor stimulation of squamous cell carcinoma cell lines, results in activation of both p38 and ERK, which in turn, leads to expression

of fibroblast growth factor-binding protein (FGF-BP), a potent angiogenic modulator [26]. Furthermore, it has been suggested that Rho proteins and the p38-MAP kinase pathway modulate IL-8 expression [63, 64]. IL-8 expression has profound biological consequences: it is a potent angiogenic, mitogenic and chemotactic factor in several malignancies including breast and prostate cancer [65–69]. Still others have suggested that FGF2, acting in an autocrine and paracrine fashion, can induce IL-6 expression through p38. In future studies we will determine whether IL-6, IL-8, and FGF2 production is also modulated by the p38 pathway in RhoC overexpressing mammary cells.

In conclusion, we have begun to identify the different signal transduction pathways involved in RhoC GTPase driven phenotypes associated with highly metastatic inflammatory breast cancer. We specifically demonstrated that anchorage independent growth is mediated via the PI3K pathway. Induction of motility and invasion are mediated through activation of the ERK and p38 arms of the MAPK pathway, and the production of VEGF is mediated primarily by p38 activation. This study provides new insight into the signal transduction pathways of an aggressive disease mediated by overexpression and activation of RhoC GTPase and suggests new potential targets for therapeutic interventions focused on the biological actions of RhoC.

Acknowledgements

This work was supported by the National Cancer Institute grant R01 CA 77612 (S.D.M.), DAMD 17-00-1-0345, from The Department of Defense, U.S. Army Breast Cancer Program (S.D.M.) and 5T32 CA 09537 and a post-doctoral fellowship (to K.L.v.G.) from the Susan G. Komen Breast Cancer Foundation. We would like to thank Ms L. Robbins for help in preparation of this manuscript.

References

1. Levine PH, Steinhorn SC, Ries LG et al. Inflammatory breast cancer: The experience of the surveillance, epidemiology, and end results (SEER) program. J Natl Cancer Inst 1985; 74: 291–7.
2. Jaiyesimi I, Buzdar A, Hortobagyi G. Inflammatory breast cancer: A review. J Clin Oncol 1992; 10: 1014–24.
3. Beahrs O, Henson D, Hutter R (eds). Manual for Staging of Cancer. Philadelphia: Lippincott 1988; 145–50.
4. Kleer cg, van Golen KL, Merajver SD. Molecular biology of breast cancer metastasis. Inflammatory breast cancer: Clinical syndrome and molecular determinants. Breast Cancer Res 2000; 2: 423–9.
5. van Golen KL, Davies S, Wu ZF et al. A novel putative low-affinity insulin-like growth factor-binding protein, LIBC (lost in inflammatory breast cancer), and RhoC GTPase correlate with the inflammatory breast cancer phenotype. Clin Cancer Res 1999; 5: 2511–9.
6. Ridley A. Membrane ruffling and signal transduction. Bioessays 1994; 16: 321–7.
7. Hall A. Rho GTPases and the actin cytoskeleton. Science 1998; 279: 509–14.
8. Ridley AJ. The GTP-binding protein Rho. Int J Biochem Cell Biol 1997; 29: 1225–9.
9. Esteve P, Embade N, Perona R et al. Rho-regulated signals induce apoptosis *in vitro* and *in vivo* by a p53-independent, but Bc12 dependent pathway. Oncogene 1998; 17: 1855–69.
10. Hall A. Small GTP-binding proteins and the regulation of the cytoskeleton. Annu Rev Cell Biol 1994; 10: 31–54.
11. Apenstrom P. Effectors for the Rho GTPases. Curr Opin Cell Biol 1999; 11: 95–102.
12. Nobes CD, Hall A. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia and filopodia. Cell 1995; 81: 53–62.
13. Helvie MA, Wilson TE, Roubidoux MA et al. Mammographic appearance of recurrent breast carcinoma in six patients with TRAM flap breast reconstructions. Radiology 1998; 209: 711–5.
14. Jimenez B, Arenda M, Esteve P et al. Induction of apoptosis in NIH3T3 cells after serum deprivation by overexpression of *rho*-p21, a GTPase protein of the *ras* superfamily. Oncogene 1995; 10: 811–6.
15. van Golen KL, Wu ZF, Qiao XT et al. RhoC GTPase, a novel transforming oncogene for human mammary epithelial cells that partially recapitulates the inflammatory breast cancer phenotype. Cancer Res 2000; 60: 5832–8.
16. van Golen KL, Wu ZF, Qiao XT et al. RhoC GTPase overexpression modulates induction of angiogenic factors in breast cells. Neoplasia 2000; 2: 418–25.
17. van Golen KL, Wu ZF, Bao LW et al. RhoC GTPase induces a motile and invasive phenotype in inflammatory breast cancer. Clin Exp Metastasis 1999; 17: 745 (Abstract #2.7).
18. Suwa H, Ohshio G, Imamura T et al. Overexpression of the *rhoC* gene correlates with progression of ductal adenocarcinoma of the pancreas. Br J Cancer 1998; 77: 147–52.
19. Genda T, Sakamoto M, Ichida T et al. Cell Motility mediated by rho and Rho-associated protein kinase plays a critical role in intrahepatic metastasis of human hepatocellular carcinoma. Hepatology 1999; 30: 1027–36.
20. Clark EA, Golub TR, Lander ES et al. Genomic analysis of metastasis reveals an essential role for RhoC. Nature 2000; 406: 532–5.
21. Takai Y, Sasaki T, Matozaki T. Small GTP-binding proteins. Physiol Rev 2001; 81: 153–208.
22. Danen EH, Sonneveld P, Sonnenberg A et al. Dual stimulation of Ras/mitogen-activated protein kinase and RhoA by cell adhesion to fibronectin supports growth factor-stimulated cell cycle progression. J Cell Biol 2000; 151: 1413–22.
23. Vojtek AB, Cooper JA. Rho family members: Activators of MAP kinase cascades. Cell 1995; 82: 527–9.
24. Arozarena I, Aaronson DS, Matallanas D et al. The Rho family GTPase Cdc42 regulates the activation of Ras/MAP kinase by the exchange factor Ras-GRF. J Biol Chem 2000; 275: 26441–8.
25. Cheng HL, Steinway ML, Russell JW et al. GTPases and phosphatidylinositol 3-kinase are critical for insulin-like growth factor-I-mediated Schwann cell motility. J Biol Chem 2000; 275: 27197–204.
26. Harris VK, Coticchia CM, Kagan BL et al. Induction of the angiogenic modulator fibroblast growth factor-binding protein by epidermal growth factor is mediated through both MEK/ERK and p38 signal transduction pathways. J Biol Chem 2000; 275: 10802–11.
27. Sodhi A, Montaner S, Patel V et al. The Kaposi's sarcoma-associated herpes virus G protein-coupled receptor up-regulates vascular endothelial growth factor expression and secretion through mitogen-activated protein kinase and p38 pathways acting on hypoxia-inducible factor 1α. Cancer Res 2000; 60: 4873–80.
28. Xiong S, Grijalva R, Zhang L et al. Up-regulation of vascular endothelial growth factor in breast cancer cells by the heregulin-beta1-activated p38 signaling pathway enhances endothelial cell migration. Cancer Res 2001; 61: 1727–32.
29. Parise LV, Lee J, Juliano RL. New aspects of integrin signaling in cancer. Semin Cancer Biol 2000; 10: 407–14.
30. Downward J. Mechanisms and consequences of activation of protein kinase B/Akt. Curr Opin Cell Biol 1998; 10: 262–7.
31. Khwaja A, Rodriguez-Viciana P, Wennstrom S et al. Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. EMBO J 1997; 16: 2783–93.
32. Ilic D, Almeida EA, Schlaepfer DD et al. Extracellular matrix survival signals transduced by focal adhesion kinase suppress p53-mediated apoptosis. J Cell Biol 1998; 143: 547–60.
33. Tamura M, Gu J, Danen EH et al. PTEN interactions with focal adhesion kinase and suppression of the extracellular matrix-dependent phosphatidylinositol 3-kinase/Akt cell survival pathway. J Biol Chem 1999; 274: 20693–703.

34. Ethier SP, Kokeny KE, Ridings JW et al. erbB family receptor expression and growth regulation in a newly isolated human breast cancer cell line. Cancer Res 1996; 56: 899–907.
35. Ethier SP. Human breast cancer cell lines as models of growth regulation and disease progression. J Mammary Gland Biol Neoplasia 1996; 1: 111–21.
36. Sartor CI, Dziubinski ML, Yu CL et al. Role of epidermal growth factor receptor and STAT-3 activation in autonomous proliferation of SUM-102PT human breast cancer cells. Cancer Res 1997; 57: 978–87.
37. Band V, Zajchowski D, Kulesa V et al. Human papilloma virus DNAs immortalize normal human mammary epithelial cells and reduce their growth factor requirements. Proc Natl Acad Sci USA 1990; 87: 463–7.
38. Stasia MJ, Vignais PV. In Abelson JM, Simon MI (eds): Methods in Enzymology. New York: Academic Press 1995; 324–7.
39. van Golen KL, Risin S, Staroselsky A et al. Predominance of the metastatic phenotype in hybrids formed by fusion of mouse and human melanoma clones. Clin Exp Metastasis 1996; 14: 95–106.
40. Albrecht-Buehler G. The phagokinetic tracks of 3T3 cells. Cell 1977; 11: 395–404.
41. Aktories K. Rho proteins: targets for bacterial toxins. Trends Microbiol 1997; 5: 282–8.
42. Chardin P, Boquet P, Madaule P et al. The mammalian G protein rhoC is ADP-ribosylated by Clostridium botulinum exoenzyme C3 and affects actin microfilaments in Vero cells. EMBO J 1989; 8(4): 1087–92.
43. Santner SJ, Dawson PJ, Tait L et al. Malignant MCF10CA1 cell lines derived from premalignant human breast epithelial MCF10AT cells. Breast Cancer Res Treat 2001; 65: 101–10.
44. Dunn SE, Torres JV, Oh JS et al. Up-regulation of urokinase-type plasminogen activator by insulin-like growth factor-I depends upon phosphatidylinositol-3 kinase and mitogen-activated protein kinase kinase. Cancer Res 2001; 61: 1367–74.
45. Cuenda A, Alessi DR. Use of kinase inhibitors to dissect signaling pathways. Methods Mol Biol 2000; 99: 161–75.
46. Wymann MP, Pirola L. Structure and function of phosphoinositide 3-kinases. Biochim Biophys Acta 1998; 1436: 127–50.
47. Lopez-Ilasaca M. Signaling from G-protein-coupled receptors to mitogen-activated protein (MAP)-kinase cascades. Biochem Pharmacol 1998; 56: 269–77.
48. Karnam P, Standaert ML, Galloway L et al. Activation and translocation of Rho (and ADP ribosylation factor) by insulin in rat adipocytes. Apparent involvement of phosphatidylinositol 3-kinase. J Biol Chem 1997; 272: 6136–40.
49. Amundadottir LT, Leder P. Signal transduction pathways activated and required for mammary carcinogenesis in response to specific oncogenes. Oncogene 1998; 16: 737–46.
50. Yujiri T, Ware M, Widmann C et al. MEK kinase 1 gene disruption alters cell migration and c-Jun NH2- terminal kinase regulation but does not cause a measurable defect in NF- kappa B activation. Proc Natl Acad Sci USA 2000; 97: 7272–7.
51. Zeigler ME, Chi Y, Schmidt T et al. Role of ERK and JNK pathways in regulating cell motility and matrix metalloproteinase 9 production in growth factor-stimulated human epidermal keratinocytes. J Cell Physiol 1999; 180: 271–84.
52. Matsumoto T, Yokote K, Tamura K et al. Platelet-derived growth factor activates p38 mitogen-activated protein kinase through a Ras-dependent pathway that is important for actin reorganization and cell migration. J Biol Chem 1999; 274: 13954–60.
53. Minden A, Lin A, Claret FX et al. Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. Cell 1995; 81: 1147–57.
54. Santibanez JF, Iglesias M, Frontelo P et al. Involvement of the Ras/MAPK signaling pathway in the modulation of urokinase production and cellular invasiveness by transforming growth factor-beta(1) in transformed keratinocytes. Biochem Biophys Res Commun 2000; 273: 521–7.
55. Royal I, Lamarche-Vane N, Lamorte L et al. Activation of cdc42, rac, PAK, and rho-kinase in response to hepatocyte growth factor differentially regulates epithelial cell colony spreading and dissociation. Mol Biol Cell 2000; 11: 1709–25.
56. Coso O, Chiariello M, Yu JC et al. The small GTP-binding proteins proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. Cell 1995; 81: 1137–46.
57. Marinissen MJ, Chiariello M, Gutkind JS. Regulation of gene expression by the small GTPase Rho through the ERK6 (p38 gamma) MAP kinase pathway. Genes Dev 2001; 15: 535–53.
58. Small JV, Kaverina I, Krylyshkina O et al. Cytoskeleton cross-talk during cell motility. FEBS Lett 1999; 452: 96–9.
59. Bishop AL, Hall A. Rho GTPases and their effector proteins. Biochem J 2000; 348 (2): 241–55.
60. Ridley AJ, Paterson H, Johnston C et al. The small GTP-binding protein rac regulates growth-factor induced membrance ruffling. Cell 1992; 70: 401–10.
61. Zondag GCM, Evers EE, ten Klooster JP et al. Oncogenic Ras downregulates Rac activity, which leads to increased Rho activity and epithelial-mesenchymal transition. J Cell Biol 2000; 149: 775–82.
62. Rak J, Mitsuhashi Y, Sheehan C et al. Oncogenes and tumor angiogenesis: differential modes of vascular endothelial growth factor upregulation in *ras*-transformed epithelial cells and fibroblasts. Cancer Res 2000; 60: 490–8.
63. Warny M, Keates AC, Keates S et al. p38 MAP kinase activation by Clostridium difficile toxin A mediates monocyte necrosis, IL-8 production, and enteritis. J Clin Invest 2000; 105: 1147–56.
64. Hippenstiel S, Soeth S, Kellas B et al. Rho proteins and the p38-MAPK pathway are important mediators for LPS-induced interleukin-8 expression in human endothelial cells. Blood 2000; 95: 3044–51.
65. Rodeck U, Becker D, Herlyn M. Basic fibroblast growth factor in human melanoma. Cancer Cells 1991; 3: 308–11.
66. Speirs V, Atkin SL. Production of VEGF and expression of the VEGF receptors Flt-1 and KDR in primary cultures of epithelial and stromal cells derived from breast tumours. Br J Cancer 1999; 80: 898–903.
67. Inoue K, Slaton JW, Eve BY et al. Interleukin 8 expression regulates tumorigenicity and metastases in androgen-independent prostate cancer. Clin Cancer Res 2000; 6: 2104–19.
68. Miller LJ, Kurtzman SH, Wang Y et al. Expression of interleukin-8 receptors on tumor cells and vascular endothelial cells in human breast cancer tissue. Anticancer Res 1998; 18: 77–81.
69. Mizuno K, Sone S, Orino E et al. Spontaneous production of interleukin-8 by human lung cancer cells and its augmentation by tumor necrosis factor alpha and interleukin-1 at protein and mRNA levels. Oncology 1994; 51: 467–71.