

Research article

WISP3 and RhoC guanosine triphosphatase cooperate in the development of inflammatory breast cancer

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Abstract

Background: Inflammatory breast cancer (IBC) is the most lethal form of locally advanced breast cancer. We found concordant and consistent alterations of two genes in 90% of IBC tumors when compared with stage-matched non-IBC tumors: overexpression of RhoC guanosine triphosphatase and loss of WNT-1 induced secreted protein 3 (WISP3). Further work revealed that RhoC is a transforming oncogene for human mammary epithelial (HME) cells. Despite the aggressiveness of the RhoC-driven phenotype, it does not quantitatively reach that of the true IBC tumors. We have demonstrated that WISP3 has tumor growth and angiogenesis inhibitory functions in IBC. We proposed that RhoC and WISP3 cooperate in the development of IBC.

Methods: Using an antisense approach, we blocked WISP3 expression in HME cells. Cellular proliferation and growth were determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-

tetrazolium bromide (MTT) assay and anchorage-independent growth in a soft agar assay. Vascular endothelial growth factor (VEGF) was measured in conditioned medium by enzyme-linked immunosorbent assay.

Results: Antisense inhibition of WISP3 in HME cells increased RhoC mRNA levels and resulted in an increase in cellular proliferation, anchorage-independent growth and VEGF levels in the conditioned medium. Conversely, restoration of WISP3 expression in the highly malignant IBC cell line SUM149 was able to decrease the expression of RhoC protein.

Conclusion: WISP3 modulates RhoC expression in HME cells and in the IBC cell line SUM149. This provides further evidence that these two genes act in concert to give rise to the highly aggressive IBC phenotype. We propose a model of this interaction as a starting point for further investigations.

Keywords: CCN proteins, motility, oncogene, tumor suppressor gene, vascular endothelial growth factor, WNT-pathway

Introduction

Inflammatory breast cancer (IBC) is the most lethal form of locally advanced breast cancer and accounts for approximately 6% of new breast cancer cases annually in the United States [1,2]. IBC has distinct clinical and pathological features. Patients present with erythema, skin nodules, dimpling of the skin (termed 'peau d'orange'), all features that develop rapidly, typically progressing within 6 months [1-4]. One salient feature of IBC that is observed in tissue

sections is that cancer cells form emboli that spread through the dermal lymphatics. The dermatotropism of IBC is believed to be responsible for the clinical signs and symptoms and probably enables effective dissemination to distant sites [2]. These observations lead us to conclude that IBC is highly invasive and that it is capable of metastases from its inception. Indeed, at the time of diagnosis, most patients have locoregional and/or distant metastatic disease [3,4]. In spite of new advances in breast cancer

FBS = fetal bovine serum; GTPase = guanosine triphosphatase; HME = human mammary epithelial; IBC = inflammatory breast cancer; MTT = 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PCR = polymerase chain reaction; RT-PCR = reverse transcriptase PCR; VEGF = vascular endothelial growth factor; WISP3 = WNT-1 induced secreted protein 3.

therapy including multimodality approaches, the 5-year disease-free survival rate is less than 45% [3,4].

Until recently, no biological markers defined the IBC phenotype. We proposed that a limited number of genetic alterations, occurring in rapid succession or concordantly, are responsible for the rapidly progressive and distinct clinical and pathological features of IBC. Using a modified version of the differential display technique and *in situ* hybridization of human tumors, we identified two genes that are consistently and concordantly altered in human IBC when compared with stage-matched non-IBC tumors: loss of WISP3 and overexpression of RhoC guanosine triphosphatase (GTPase) [5].

WNT-1 induced secreted protein 3 (WISP3) is a member of the CCN family of proteins, which have important biological functions in normal physiology as well as in carcinogenesis [6–8]. We found that WISP3 has growth and angiogenesis inhibitory functions in IBC *in vitro* and *in vivo* [9]. RhoC GTPase is a member of the Ras superfamily of small GTPases. Activation of Rho proteins leads to assembly of the actin–myosin contractile filaments into focal adhesion complexes that bring about cell polarity and facilitate motility [10–12]. Our laboratory has characterized RhoC as a transforming oncogene for human mammary epithelial (HME) cells; its overexpression results in a highly motile and invasive phenotype that recapitulates the IBC phenotype. Predicated on the high rate of concordance of RhoC and WISP3 changes in IBC, we propose that these two genes cooperate to determine this highly metastatic, unique breast cancer phenotype.

Materials and methods

Cell culture

The derivation of the SUM149 cell line has been described previously by Ethier et al [13]. This cell line was developed from a human primary IBC and has lost WISP3 expression [9]. HME cells were immortalized with human papilloma virus E6/E7 and were characterized as being keratin 19 positive, ensuring that they are from the same differentiation lineage as the SUM149 IBC tumor cell line [14,15]. MCF10A cells are spontaneously immortalized human mammary epithelial cells. Cells were cultured in Ham's F-12 medium supplemented with 5% fetal bovine serum (FBS), hydrocortisone (1 µg/ml), insulin (5 µg/ml), fungizone (2.5 µg/ml), gentamycin (5 µg/ml), penicillin (100 U/ml) and streptomycin (10 µg/ml) at 37°C under 10% CO₂.

Construction of expression vectors and stable transfections

Total RNAs were isolated from HME cells with a Trizol kit (Life Technologies, Inc, Gaithersburg, MD). First-strand cDNA synthesis was performed by using 1 µg of total RNA with AMV reverse transcriptase (Promega, Madison,

WI) and oligo(dT) as a primer. A 2 µl portion of the reaction mixture was used for amplification by polymerase chain reaction (PCR). Human WISP3 cDNA was amplified by PCR with the forward and reverse primers 5'-ACGAATTCAATGAACAAGCGGCG-3' and 3'-GCGAATTCTTTACAGAATCTTG-5', respectively, under the following conditions: denaturing for 1 min at 94°C, annealing for 1 min at 58°C, and elongation for 1 min at 72°C, for 35 cycles. PCR products were cloned into pGEM-T Easy vector (Promega). The 1.1 kb full-length cDNA encoding WISP3 was excised by *EcoRI* and subcloned into the *EcoRI* site of pFlag-CMV4 vector (Sigma, St Louis, MO). The insert was confirmed by DNA sequencing. The plasmids were purified. Subsequently, the SUM149 cells were transfected with pFlag-WISP3 sense (SUM149/WISP3), and HME cells were transfected with pFlag-WISP3 anti-sense (HME/AS WISP3). MCF10A cells were stably transfected with full-length RhoC cDNA (MCF10A/RhoC). pFlag control vectors were used as controls (FuGene TM 6 transfection reagent; Roch–Boehringer-Mannheim, Germany). Transfectants were selected in the medium containing 150 µg/ml G418. The cells surviving during selection were expanded and maintained in the selected medium.

Reverse transcriptase PCR (RT-PCR) analysis

Total RNA (1 µg) from HME/AS WISP3 clones and empty vector controls were reverse-transcribed with Superscript reverse transcriptase (Invitrogen) using oligo(dT) and random hexanucleotide primer for first-strand cDNA synthesis. PCRs were performed directly on 1 µl of first-strand cDNA using 500 nmol of each of the following gene-specific primers: WISP3, 5'-ATGCAGGGGCTCCTCTTCTGC-3' (forward primer) and 5'-ACTTTTCCCCATTGCTTG-3' (reverse primer); RhoC, 5'-ATGGCTGCAATCCGAAAAG-3' (forward primer) and 5'-GATCTCAGAGAATGGGACAGC-3' (reverse primer); GAPDH, 5'-CGGAGTCAACGGATTG-GTTCGTAT-3' (forward primer) and 5'-AGCCTTCTCCATG-GTGGTGAAGAC-3' (reverse primer). The 100 µl reaction volume consisted of 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, and deoxynucleotide triphosphates (each at 200 µM). PCR was performed for initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min), and extension (72°C, 1 min) with 5 units of *Taq* polymerase (Invitrogen). This was followed by a final extension step at 72°C for 10 min. The products were analyzed on 1% agarose gels stained with ethidium bromide and detected with ultraviolet illumination.

Western immunoblots

Western immunoblots were performed with polyclonal anti-RhoC and anti-WISP3 antibodies. Cultured cells were washed in ice-cold phosphate-buffered saline, lysed in lysis buffer (10% glycerol, 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1% Nonidet P40, 2 mM MgCl₂, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mM phenylmethylsulphonyl

fluoride) on ice for 5 min, and then centrifuged for 5 min at 4°C. Cleared lysates (each containing 50 µg of protein) were subjected to SDS-polyacrylamide-gel electrophoresis and transferred to poly(vinylidene difluoride) membrane. Western blots were performed as described previously, with anti-RhoC rabbit polyclonal antibody and anti-β-actin goat antibody (Sigma) at dilutions of 1:1500 and 1:2000, respectively [16].

Anchorage-independent growth

For studies of anchorage-independent growth we performed soft agar assays on stable clones of HME/AS WISP3, HME/Flag, and SUM149 cells. Each well of a six-well plate was first layered with 0.6% agar diluted with 10% FBS-supplemented Ham's F-12 medium complete with growth factors. The cell layer was then prepared by diluting agarose to concentrations of 0.3% and 0.6% with 10³ cells in 2.5% FBS-supplemented Ham's F-12/1.5 ml/well. Plates were maintained at 37°C under 10% CO₂ for 3 weeks. Colonies 100 µm or more in diameter were counted under the microscope with a grid.

Monolayer growth rate

Monolayer culture growth rate was determined by qualitative measurement of the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) to a water-insoluble formazan by viable cells. In all, 3000 cells obtained for HME/AS WISP3 clones, HME/Flag, and HME wild-type cells, suspended in 200 µl of culture medium, were plated in 96-well plates and grown under normal conditions. Cultures were assayed at 0, 2, 4, 6, and 8 days by the addition of MTT and incubation for 1 hour at 37°C. The MTT-containing medium was aspirated and 100 µl of dimethyl sulphoxide (Sigma) was added to lyse the cells and solubilize the formazan. Optical densities of the lysates were determined on a Dynatech MR 5000 microplate reader at 595 nm.

Analysis of vascular endothelial growth factor

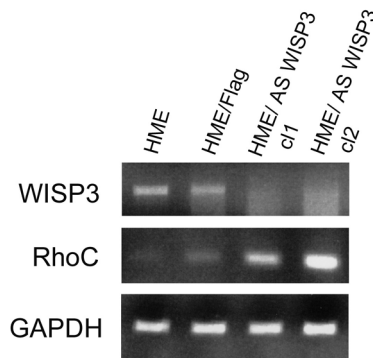
Conditioned medium was generated by incubating HME/AS WISP3 cells, HME/Flag cells, and SUM149 cells in serum-free medium. After 3 days the medium was collected, cleared of cell debris by centrifugation, concentrated approximately 10-fold through a Centrplus YM-10 column (Millipore, Bedford, MA). The levels of vascular endothelial growth factor (VEGF), which is a factor known to be secreted by IBC, were measured in the cell culture supernatants by enzyme-linked immunosorbent assay, as described previously [9,17].

Results

Inhibition of WISP3 increases RhoC mRNA levels in immortalized HME cells and induces proliferation, anchorage-independent growth, and VEGF production

To study the effects of inhibition of WISP3 expression on the phenotype of HME cells, we established clones of

Figure 1



Inhibition of WISP3 in human mammary epithelial (HME) cells results in an increase in RhoC transcript levels. Reverse transcriptase polymerase chain reaction was conducted on vector and HME cells that have inhibition of WISP3 expression using full-length WISP3 antisense mRNA. HME/AS WISP3 cells showed increased levels of RhoC transcript in comparison with controls.

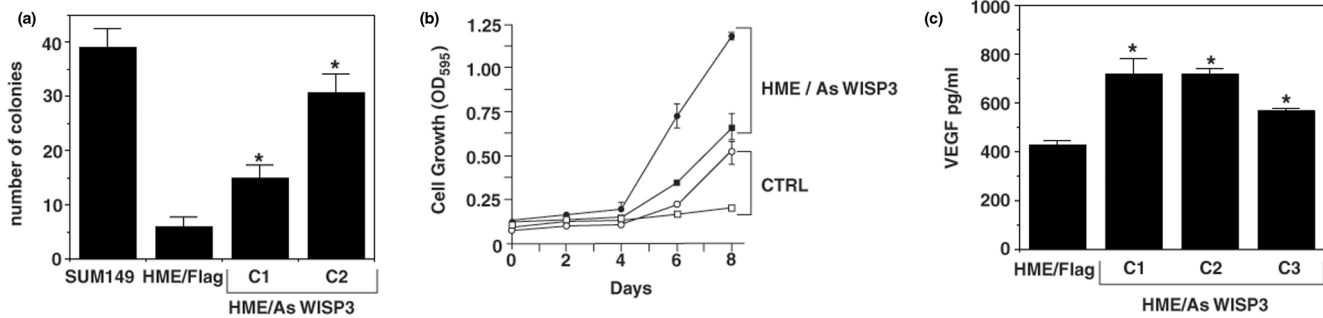
HME cells stably transfected with antisense WISP3 constructs (HME/AS WISP3). Effective inhibition of WISP3 expression was confirmed by RT-PCR (Fig. 1). Inhibition of WISP3 expression in HME cells resulted in increased expression of RhoC transcript in comparison with HME cells transfected with the control empty vector (Fig. 1). After 14 days of growth in soft agar, inhibition of WISP3 expression in HME cells resulted in a significant increase in the number of colonies formed in comparison with the empty vector control (*t*-test, $P < 0.05$ for both clones; Fig. 2a). Inhibition of WISP3 expression resulted in an increase in cellular proliferation (*t*-test, $P < 0.05$; Fig. 2b).

Previously, we had shown that restoration of WISP3 expression in an IBC cell line decreases the production of VEGF, a major pro-angiogenic factor secreted by IBC [9]. To determine the effect of WISP3 inhibition on the secretion of VEGF, we measured the concentration of VEGF in the conditioned medium of the stably transfected HME/AS WISP3 cells. Figure 2c shows that inhibition of WISP3 expression resulted in increased levels of VEGF in the conditioned medium (*t*-test, $P < 0.05$ for all clones).

Restoration of WISP3 expression in SUM149 cells decreases RhoC expression

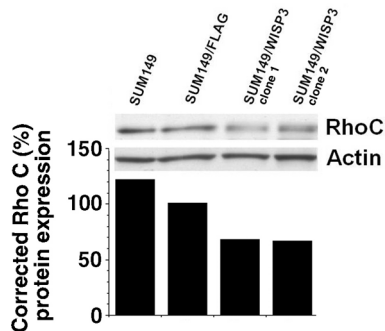
Because decreased expression of WISP3 in HME cells induced a significant increase in RhoC expression and some features of RhoC induced functional changes including anchorage-independent growth and production of VEGF, we sought to determine whether restoration of WISP3 expression in the SUM149 IBC cell line, which has lost WISP3 expression in the wild type, has an effect in RhoC expression. To test this, we stably transfected WISP3 in SUM149 cells and measured RhoC expression

Figure 2



Inhibition of WISP3 induces anchorage-independent growth, proliferation and secretion of vascular endothelial growth factor (VEGF) in human mammary epithelial (HME) cells. **(a)** Inhibition of WISP3 expression in HME cells. HME cells greatly increased the number of colonies formed in soft agar in comparison with empty vector control (HME/Flag; *t*-test, $P < 0.05$ for both clones). **(b)** Effect of inhibition of WISP3 expression on the proliferation of HME cells was studied with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The stable HME/AS WISP3 cells have a significant increase in the proliferation rate in comparison with the empty vector control. Results are expressed as means \pm SEM of three independent experiments. In all, 3000 cells were assessed in each plate (*t*-test, $P < 0.05$). **(c)** Increase in VEGF measured by enzyme-linked immunosorbent assay, as a result of inhibition of WISP3 expression in HME cells. Results are expressed as means \pm SEM; *t*-test, $P < 0.05$ for all clones.

Figure 3



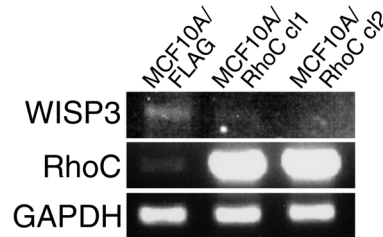
Restoration of WISP3 expression in SUM149 inflammatory breast cancer cells decreases RhoC protein expression. Western immunoblot of cell culture of SUM 149 cells, empty vector control (SUM149/Flag), and two WISP3-expressing clones with antibodies against RhoC and actin. Gels were scanned and pixel intensity values were obtained. Values for RhoC were corrected for loading by dividing the RhoC pixel intensity by the actin pixel intensity.

by Western blotting. Restoration of WISP3 expression in SUM149 cells resulted in a decrease in RhoC protein expression in comparison with the empty vector control (Fig. 3). To investigate whether the relationship between WISP3 and RhoC expression is reciprocal, we developed MCF10A cells stably transfected with RhoC. These cells showed a 2.5-fold decrease in WISP3 mRNA expression (Fig. 4).

Discussion

Our previous work showed that the overexpression of RhoC GTPase and the loss of WISP3 expression are alterations that occur concordantly, more often in IBC than

Figure 4



RhoC overexpression in MCF10A cells results in a decrease in WISP3 mRNA level. Reverse transcriptase polymerase chain reaction was conducted on vector and MCF10A cells stably overexpressing RhoC. RhoC-overexpressing cells had decreased levels of WISP3 mRNA.

in slow-growing locally advanced breast cancers. WISP3 loss was found in concert with RhoC GTPase overexpression in 90% of archival patient samples of IBC, but rarely in stage-matched non-IBC tumors. Our laboratory further demonstrated that RhoC GTPase is a transforming oncogene for HME cells and that WISP3 has tumor inhibitory functions in IBC. However, neither alteration occurring in isolation seems to be sufficient to develop the full-blown, highly malignant IBC phenotype. Here we postulate that dysregulation of WISP3 might upregulate RhoC GTPase and thus enhance the aggressiveness of the phenotype that results when these two alterations are present.

We have shown that overexpression of RhoC GTPase in immortalized HME cells produced a striking tumorigenic effect that, for the most part, recapitulates the phenotype of the SUM149 IBC cell line. HME cells stably transfected with RhoC exhibited greatly increased growth under anchorage-independent conditions [15]. HME cells over-

expressing RhoC produced up to 100-fold more colonies than the controls, about 60% of the level of colony formation of the SUM149 IBC cell line. RhoC overexpression induced motility and invasion in HME cells, and markedly induced the production of angiogenic mediators including VEGF [17]. The HME/RhoC transfectants formed tumors when injected into the mammary fat pad of athymic nude mice [15].

Importantly, restoration of WISP3 in SUM149 cells ameliorated these features of the malignant phenotype. The SUM149/WISP3⁺ cells exhibited decreased growth *in vitro* and *in vivo* in comparison with SUM149 cells transfected with the empty vector. The invasiveness of SUM149 cells was greatly decreased by restoring WISP3 expression. We also found that WISP3 markedly decreased the concentration of angiogenic mediators in the conditioned medium, especially VEGF, basic fibroblast growth factor, and interleukin-6 [9]. Given the high specificity of WISP3 and RhoC alterations in IBC and their interrelated functions in tumorigenesis, we propose that they cooperate in the development of IBC.

Using an antisense approach, inhibition of WISP3 expression in HME cells resulted in a threefold increase of RhoC GTPase transcript levels. The HME/AS WISP3 cells also exhibited increased cellular proliferation and anchorage-independent growth in soft agar. The HME/AS WISP3 cells produced significantly more colonies in soft agar in comparison with the control cells, an average of 58% of the level of colonies formed by the SUM149 IBC cells. HME/AS WISP3 cells also exhibited decreased production of VEGF in the conditioned medium.

The relationship between RhoC and WISP3 expression seems to be reciprocal. Restoration of WISP3 expression in SUM149 cells, which have lost WISP3 in the wild-type state, induced a 1.5-fold decrease in RhoC GTPase expression. These results are intriguing because changes in expression in Rho proteins by subtle factors such as 1.5–1.8 can be sufficient to modulate cellular behavior. Overexpression of RhoC in spontaneously immortalized HME cells, MCF10A, resulted in a 2.5-fold decrease in WISP3 mRNA expression.

In summary, overexpression of RhoC GTPase and loss of WISP3 are key genetic alterations in the development of IBC, and they have complementary functions. RhoC GTPase has a primary role in motility, invasion, and angiogenesis [15,17]. WISP3 has a pivotal role in tumor growth, invasion, and angiogenesis [9]. Here we have further strengthened the evidence that these genes cooperate in the development of IBC, because WISP3 expression modulates the expression of RhoC GTPase and its functions.

Conclusion

IBC is the most lethal form of locally advanced breast cancer, with a 5-year disease-free survival of less than 45%. Our work focused on determining the genetic alterations that result in this aggressive breast cancer phenotype. Previously, we have found that RhoC and WISP3 are consistently and concordantly altered in IBC tissues. RhoC functions as an oncogene, and WISP3 as a tumor suppressor gene. Here we provide evidence supporting the hypothesis that these two genes act in concert to give rise to the highly aggressive IBC phenotype. We propose a model of this interaction as a starting point for further investigations.

Competing interests

None declared.

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