WISP3 (CCN6) Is a Secreted Tumor-Suppressor Protein that Modulates IGF Signaling in Inflammatory Breast Cancer

Celina G. Kleer*, Yanhong Zhang*, Quintin Pan† and Sofia D. Merajver†

Departments of *Pathology and †Internal Medicine, Division of Hematology and Oncology, University of Michigan Medical Center, Ann Arbor, MI, USA

Abstract

Inflammatory breast cancer (IBC) is the most lethal form of locally advanced breast cancer. We have found that WISP3 is lost in 80% of human IBC tumors and that it has growth- and angiogenesis-inhibitory functions in breast cancer in vitro and in vivo. WISP3 is a cysteine-rich, putatively secreted protein that belongs to the CCN family. It contains a signal peptide at the N-terminus and four highly conserved motifs. Here, for the first time, we investigate the function of WISP3 protein in relationship to its structural features. We found that WISP3 is secreted into the conditioned media and into the lumens of normal breast ducts. Once secreted, WISP3 was able to decrease, directly or through induction of other molecule(s), the IGF-1–induced activation of the IGF-IR, and two of its downstream signaling molecules, IRS1 and ERK-1/2, in SUM149 IBC cells. Furthermore, WISP3 containing conditioned media decreased the growth rate of SUM149 cells. This work sheds light into the mechanism of WISP3 function by demonstrating that it is secreted and that, once in the extracellular media, it induces a series of molecular events that leads to modulation of IGF-IR signaling pathways and cellular growth in IBC cells.

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Introduction

Inflammatory breast cancer (IBC) is the most lethal form of locally advanced breast cancer [1,2]. It is also a very distinct clinical and pathological type of carcinoma. Clinically, patients present with what has been classically termed “peau d’orange,” characterized by skin thickening and dimpling, also with nodularity, erythema, and, often, nipple retraction [1–4]. IBC is highly angiogenic and angioinvasive. Clusters of malignant cells invade the dermal lymphatics, forming tumor emboli that likely cause the clinical symptoms, and disseminate to distant sites [1].

In our previous work, we found that WISP3 is lost in 80% of human IBC tumors and is a key genetic determinant of the IBC phenotype [5]. WISP3 has growth-, invasion-, and angiogenesis-inhibitory functions in IBC in vitro and in vivo [6]. WISP3 is a member of the CCN family of proteins, which also includes connective tissue growth factor (CTGF), Cyr61, Nov, WISP1, and WISP2 [7,8]. A putatively secreted protein with a secretory signal peptide at the NH2 terminus, WISP3 contains 36 conserved cysteine residues that are organized into four highly conserved modules: 1) a motif associated with insulin-like growth factor binding protein (IGFBP) (GGGCCXXC); 2) a von Willebrand type C–like motif; 3) a thrombospondin 1 module; and 4) a carboxyl-terminal domain putatively involved in dimerization [8,9]. The role of each of these conserved domains in the function of the CCN proteins, in general, and of WISP3, in particular, remains to be elucidated.

IGF-I and its major receptor, IGF-IR, play an important role in normal breast biology and in the development of breast cancer [10–13]. A large body of work implicates the IGF family in breast cancer progression. High concentrations of IGF-I in serum are associated with increased mammographic density (one of the strongest predictors of breast cancer risk), and also reliably predict increased breast cancer risk specifically in premenopausal women [14]. In vitro, IGF-I is a strong mitogen for human breast cancer cells and has been found in the epithelial and stromal component of breast cancers [13]. High expression of IGF-IR has been demonstrated in most primary human breast cancers when compared to normal or benign breast tissues, and hyperactivation of IGF-IR in breast cancer has been linked to increased radioresistance and cancer recurrence at the primary site [13,15,16]. High levels of IRS-1, a major signaling molecule downstream of the IGF-IR, correlate with tumor size and shorter disease-free survival in ER+ breast cancer patients [17,18]. Based on the protein sequence of WISP3 and the important role of IGF signaling in breast cancer, we hypothesized that WISP3 is secreted into the extracellular medium and that the growth-inhibitory effect of...
WISP3 in IBC may be dependent, at least in part, on modulation of IGF-I signaling. To test this hypothesis, we investigated the downstream effects of WISP3 starting at the IGF-IR receptor and signaling pathway. Here, we demonstrate that WISP3 is a secreted protein and that, once in the conditioned media, it can effectively modulate IGF-IR activation and its signaling cascade and the cellular growth of IBC cells.

Materials and Methods

Cell Culture and Transfections

SUM149 cells derive from a primary IBC that has lost WISP3 expression [6,19]. SUM149 cells and their transfectants were cultured in Ham’s F-12 media supplemented with 5% fetal bovine serum (FBS), hydrocortisone (1 µg/ml), insulin (5 µg/ml), fungizone (2.5 µg/ml), gentamycin (5 µg/ml), and penicillin/streptomycin (100 µg/ml each), at 37°C under 10% CO2. HEK-293 cells derived from human embryonic kidney epithelial cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS. SUM149 and HEK-293 cells were transfected with HIS-tagged (pcDNA 3.1/V5-HIS TOPO TA expression vector; Invitrogen, Carlsbad, CA) and Flag-tagged (pFlag-CMV vector; Sigma, St. Louis, MO) full-length WISP3 cDNA, and clonal cell lines were established as described previously [6]. Control cell lines were generated by transfecting the SUM149 cell line that does not express WISP3 (SUM149 wild type), and a cell line that expresses high levels of WISP3 (SUM149 cell line stably transfected with WISP3) and from a tumor Xenograft. Positive and negative controls were tumor xenografts derived from cell lines shown to express high levels of WISP3 (SUM149 IBC cells previously characterized with a loss of WISP3 expression) and from a cell line that does not express WISP3 (SUM149 wild type) respectively.

Immunoprecipitation and Western Blotting

Cells were lysed in lysis buffer composed of 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EGTA, 1 mM Na3VO4, 1 mM PMSF, and 1 µg/ml aprotime. The lysates were clarified by centrifugation at 14,000 g for 10 minutes. A total of 500 µg of cell lyses was incubated with 1 µg/ml anti–IGF-IR mAb (Calbiochem, San Diego, CA) overnight at 4°C. Immune complexes were precipitated by adding 50 µl of protein A/G plus agarose bead slurry for 2 hours. The agarose beads were collected and washed three times with ice-cold lysis buffer, and resuspended in 25 µl of Laemmli sample buffer for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Fifty micrograms of protein extract was separated by SDS-PAGE and transferred onto a PVDF membrane (Amersham Pharmacia Biotech). The precipitated IGF-IR was detected with anti–IGF-IR β subunit polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA). Tyrosine phosphorylation of immunoprecipitated IGF-IR was assessed with anti–phosphotyrosine mAB PY20 (Transduction Laboratories, Lexington, KY). Total IGF-IR, phosphorylated and total IRS1, and ERK-1/2 were measured with appropriate antibodies (Transduction Laboratories; Upstate Biotechnology Inc.). WISP3 expression was confirmed by Western blot using a polyclonal anti–WISP3 antibody (gift from Dr. Warman) and an antibody against the HIS tag (Invitrogen). The protein bands were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). All experiments were repeated at least three times and the optical density of the bands was quantified by densitometry (Scio Image software for Win 95/98, version 0.4). Statistical analysis was performed using 95% confidence intervals for the estimates of the means. A P value of <.05 was considered statistically significant.

Effect of WISP3 in the Proliferation of SUM149 cells

SUM149 cells were plated in 96-well tissue culture plates at a density of 5 × 10^4 cells/ml in Ham’s F-12 media with 5% FBS. One hundred microliters of serum-free medium was added for 24 hours. Ten-fold concentrated WISP3 and control conditioned media were added in the presence and absence of IGF-I simulation as described above. MTT reagents were added 24 hours later according to the manufacturer’s protocol (Sigma), and the plate was read at a wavelength of 595 nm. The experiment was performed in triplicate.

Human Breast Tissues and Immunohistochemistry

WISP3 protein expression was studied by immunohistochemistry in normal human breast tissues obtained from 10 reduction mammoplasty procedures. Immunohistochemical analysis was performed by using a polyclonal anti-WISP3 antibody at 1:500 dilution with overnight incubation and microwave antigen retrieval [20]. The detection reaction followed the Dako Envision+ System Peroxidase kit protocol (Dako, Carpinteria, CA). Diaminobenzidine was used as chromogen and hematoxylin was used as counterstain. Positive and negative controls were tumor xenografts derived from cell lines shown to express high levels of WISP3 (SUM149 cell line stably transfected with WISP3) and from a cell line that does not express WISP3 (SUM149 wild type) respectively.

Results

WISP3 Protein Is Secreted by Human Breast Epithelial Cells

WISP3 protein contains a multimodular structure with a secretory signal peptide at the N-terminus. To investigate whether WISP3 is secreted by breast epithelial cells, SUM149 IBC cells previously characterized with a loss in WISP3 expression were stably transfected to express full-length WISP3. Conditioned media from SUM149/ WISP3–overexpressing clones were collected and
detected for WISP3 by Western blot analysis using a polyclonal anti–WISP3 antibody. WISP3 protein was detected in the media of SUM149-expressing WISP3 (SUM149/WISP3), and not in the media of empty vector–transfected SUM149 cells (SUM149/Flag) (Figure 1A). In order to explore these results from a different perspective, we performed transient transfections of WISP3 in SUM149 cells using a HIS\(^{\text{c}}\)-tagged full-length WISP3 expression vector. In this case, WISP3 protein was detected in the conditioned media using both anti–WISP3 antibody and anti–HIS antibody (data not shown). To specifically address whether WISP3 would be detected in the conditioned media of a nonmammary cell, we repeated these experiments with the HEK293 cell line, derived from human embryonic kidney epithelial cells. WISP3 was detected in the conditioned media of these cells and not in the conditioned media of the empty vector controls (Figure 1B).

**In situ** expression of WISP3 protein was determined by immunohistochemical analysis of normal breast tissues derived from reduction mammoplasty procedures. In all 10 tissues examined, WISP3 protein was expressed at low levels in the cytoplasm of normal epithelial cells from ducts and acini and, interestingly, was present in the luminal secretions of ducts and lobules (Figure 2A). Xenografts derived from wild-type SUM149 cells (WISP3\(^{-}\)) and from SUM149/WISP3\(^{+}\) cells were used as negative and positive controls, respectively (Figure 2B and C).

**WISP3 Containing Conditioned Media Reduces IGF-I–Induced IGF-IR Activation and Signaling Pathways**

The effect of WISP3 on the activation of the IGF-I signaling pathway was studied in SUM149 cells derived from a primary IBC [5,6,19,21]. The activation of the IGF signaling

![Figure 1.](image1.png)

**Figure 1.** WISP3 protein is secreted and detected in the conditioned media. (A) Western immunoblot using anti–WISP3 polyclonal antibody detects WISP3 protein in the conditioned media of SUM149 cells transfected with WISP3 full-length cDNA. WISP3 protein is not detected in the conditioned media of SUM149 cells transfected with the empty vector. (B) Western immunoblot of the conditioned media of HEK-293 cells transfected with WISP3, detected using an anti–HIS antibody. WISP3 is not detected in the conditioned media of the control cells.

![Figure 2.](image2.png)

**Figure 2.** In situ expression of WISP3 protein in normal breast tissues. (A) Immunohistochemical analysis of normal breast tissues using a polyclonal anti–WISP3 antibody shows that WISP3 protein is expressed at low levels in the cytoplasm of normal epithelial cells and in the luminal secretions of ducts and acini (arrows) (× 200). (B and C) Xenografts derived from wild-type SUM149 and SUM149/WISP3 cells were used as negative and positive controls, respectively (× 400).
cascade plays a central role in breast cancer development and progression. To investigate whether the presence of WISP3 in the conditioned media has an effect on IGF-IR signaling pathways in IBC cells, phosphorylation of IGF-IR, IRS1, and ERK-1/2 was determined in wild-type SUM149 cells in the presence or absence of WISP3 in the conditioned media. Experiments were carried out under baseline conditions without addition of IGF-I and after stimulation with IGF-I. In the presence of WISP3 in the conditioned media, SUM149 cells exhibited decreased IGF-IR phosphorylation (Figure 3). The effect of WISP3 in the phosphorylation of the IGF-IR was evident in the presence of IGF-I stimulation because WISP3 was able to ameliorate the effect of IGF-I stimulation on the activation of the IGF-IR (Figure 3). Similarly, WISP3 conditioned media decreased the IGF-I–induced IRS1 and ERK-1/2 phosphorylation (Figures 4 and 5).

Figure 3. WISP3 decreases IGF-I–induced phosphorylation of the IGF-IR. (A) Western blot analysis of SUM149 cell lines bathed in WISP3+ and control (WISP3−) conditioned media. The experiment was carried out under baseline conditions (without IGF-I) and after stimulation with 20 ng/ml IGF-I. The IGF-IR was precipitated from 500 μg of protein lysate with an anti–IGF-IR mAb and subsequently detected by immunoblot with an anti–IGF-IR/12 subunit polyclonal Ab. Tyrosine phosphorylation of immunoprecipitated IGF-IR was assessed with an anti–phosphotyrosine mAb PY20. (B) Relative protein levels of IGF-IR phosphorylation normalized for total IGF-IR. Blots were scanned and the pixel intensity measured using Scn Image program. Results are expressed as mean ± SEM of three independent experiments. (C) The differences in the percent activation of IRS-1 were quantitated. The difference in the corrected IRS1 phosphorylation under baseline conditions and after IGF-I stimulation was calculated for each cell line and results were normalized using the difference in IRS1 phosphorylation in the absence of IGF-I stimulation as reference. WISP3-rich conditioned media was able to decrease the phosphorylation of IRS-1 triggered by IGF-I (t test, P < .05).

Figure 4. WISP3 decreases IGF-I–induced phosphorylation of IRS1. (A) Western blot analysis of SUM149 cell lines bathed in WISP3+ and control WISP3-conditioned media. The experiment was carried out under baseline conditions (without IGF-I) and after stimulation with IGF-I. The expression of phosphorylated IRS1 was detected by Western blot using a polyclonal antibody against phosphorylated IRS1. The Western blot was stripped and probed with an antibody against total IRS1. (B) Relative protein levels of IRS1 phosphorylation normalized for total IRS1. Blots were scanned and the pixel intensity measured. Results are expressed as mean ± SEM of three independent experiments. (C) The differences in the percent activation of IRS-1 were quantitated. The difference in the corrected IRS1 phosphorylation under baseline conditions and after IGF-I stimulation was calculated for each cell line and results were normalized using the difference in IRS1 phosphorylation in the absence of IGF-I stimulation as reference. WISP3-rich conditioned media was able to decrease the phosphorylation of IRS-1 triggered by IGF-I (t test, P < .05).

WISP3 Containing Conditioned Media Reduces the Growth of IBC Cell Lines

After establishing that the presence of WISP3 in the conditioned media was able to modulate IGF-I signaling pathways, its effect on cellular proliferation was determined in the presence and absence of IGF-I stimulation. SUM149 cells bathed in the WISP3 conditioned media had significantly lower growth rates than the control SUM149 cells bathed in control (WISP3-deficient) conditioned media, both in the presence and absence of IGF-I stimulation (t test, P < .05; Figure 6).

Discussion

We have previously demonstrated that WISP3 is lost in 80% of IBC tumors and that it has tumor-suppressor functions in IBC [5,6]. Studies on the SUM149 IBC cell line showed that restoration of WISP3 expression has potent growth- and
angiogenesis-inhibitory functions in vitro and in vivo [6]. Restoration of WISP3 resulted in a significant decrease in anchorage-independent growth in soft agar and cellular proliferation, as well as a drastic decrease in the invasive capabilities of the SUM149 cells, which are highly invasive in their wild-type state. Furthermore, restoration of WISP3 expression in SUM149 cells resulted in a biologically relevant decrease in the level of angiogenic factors (VEGF, bFGF, and IL-6) in the conditioned media of the cells. In vivo, restoration of WISP3 expression in SUM149 cells caused a drastic decrease in tumor volume and rate of tumor growth when injected in nude mice [6]. Taken together, this body of work had strongly supported a tumor-suppressor role for WISP3 in mammary tumor progression. In the present study, we sought to discover the molecular mechanisms underlying the tumor-suppressor function of WISP3.

WISP3 belongs to the CCN family of proteins, which are highly conserved, putatively secreted proteins with important roles in development during chondrogenesis and skeletogenesis [7]. The CCN proteins have been recently also implicated in carcinogenesis [7,22–26]. It is not well understood, however, how the functions of the CCN proteins in development relate to their role in cancer. Moreover, their expression during tumorigenesis cannot be generalized across different tissue types. This may be due to tissue-specific functions of the CCN proteins, perhaps mediated by their multimodular structure and the presence of different affinities for binding partners and ligands in different tissues [7]. The presence of different receptors and differential processing of the CCN proteins (e.g., cleavage by proteases) may account also for their diverse functions in different tissues. In this paper, we focused on determining whether WISP3 is secreted into the conditioned media and its relationship to IGF signaling pathways.

Figure 5. WISP3 decreases IGF-I–induced phosphorylation of ERK-1/2. (A) Western blot analysis of SUM149 cell lines bathed in WISP3– and control (WISP3+) conditioned media, under baseline conditions (without IGF-I) and after stimulation with IGF-I. The expression of ERK-1/2 was detected by Western blot using a polyclonal antibody against phosphorylated ERK-1/2. The blot was stripped and probed with an antibody against -actin. (B) Relative protein levels of ERK-1/2 phosphorylation normalized using actin. Blots were scanned and the pixel intensity measured. Results are expressed as mean ± SEM of three independent experiments. (C) Quantitation of the differences in the percent activation of ERK-1/2. The difference in the corrected ERK-1/2 phosphorylation under baseline conditions and after IGF-I stimulation was calculated for each cell line. Results were normalized using the difference in ERK-1/2 phosphorylation in the absence of IGF-I stimulation as reference. WISP3-rich conditioned media ameliorated the phosphorylation of ERK 1 and ERK 2 induced by IGF-I stimulation (t test, P < .05).

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Analysis of the protein sequence of WISP3 revealed that it contains a signal peptide at the N-terminal region that may participate in the secretion of the protein into the extracellular media [7–9]. Indeed, by Western blot, using two different specific antibodies, we were able to detect WISP3 protein in the conditioned media of SUM149 and HEK293 cells transfected with WISP3. Furthermore, consistent with these results, by immunohistochemical analysis, WISP3 protein was detected in the secretions accumulated in the lumens of ducts and lobules in normal breast tissues. The fact that WISP3 is secreted and present in the conditioned media (thereby alluding to its stability in solution) led us to the hypothesis that it may directly or indirectly regulate IGF signaling.

Although the signaling pathways that are required for the effects of IGF-I in breast cancer have not been completely elucidated, the contribution of IGF-I–induced IGF-IR
activation appears to be critical in hormone-dependent and -independent breast cancer [27–30]. IGF-I is locally released by breast cancer cells and stromal fibroblasts, and it is involved in autocrine and paracrine stimulation of the mammary epithelium [31]. In breast cancer cells, when IGF-I binds IGF-IR, signaling occurs mainly through activation of IRS-1 and RAS-dependent phosphorylation of MAP kinase with subsequent activation of nuclear transcription factors [32,33]. IGF-I signaling promotes cell growth, survival, and motility of breast cancer cells, as well as resistance to therapeutic interventions [10–12,14–18]. We hypothesized that expression of WISP3 could result in a series of molecular events that leads to the modulation of IGF-IR activation and downstream signaling. Contributing to this hypothesis is the fact that we have shown that WISP3 is secreted into the media where it has the opportunity to directly or indirectly modulate the strength of IGF signaling. Indeed, in the presence of IGF-I, WISP3 containing conditioned media decreased IGF-IR phosphorylation and the phosphorylation of two main downstream IGF-IR signaling molecules, IRS1 and ERK-1/2. This inhibition was not evident under baseline conditions, without stimulation with IGF-I. Our experiments thus show that even relatively small concentrations of WISP3 secreted by WISP3-transfected cells are able to modulate, directly or indirectly, IGF-I signaling in the setting of IGF-I stimulation.

A major growth-regulatory IGF-IR downstream pathway that regulates breast cancer growth and survival converges on ERK-1/2 cascade [34]. We observed a decrease in ERK-1/2 phosphorylation by addition of WISP3 containing conditioned media in the presence of IGF-I stimulation. ERK-1/2 influence chromatin remodeling and activation of gene expression, leading to enhanced cellular proliferation and decreased apoptosis [35–37]. Specifically, ERK-1/2 have been shown to activate the transcription of key genes involved in cell cycle progression including cyclin D1 and cyclin E. We have shown previously that restoration of WISP3 expression in the highly malignant SUM149 IBC cell line markedly decreased the levels of cyclin E and PCNA, a reliable marker of cellular proliferation [6].

The mechanism whereby WISP3 may modulate IGF-IR activation in the presence of IGF-I remains to be elucidated. WISP3 contains a highly conserved motif (GCGCCXXC) characteristic of IGFBPs, which may provide the proper protein folding to interact with IGF-like ligands, thereby enabling interference with IGF signaling. Although initial studies reported that two other CCN proteins, CTGF (CTGF) and Nov, specifically bind to IGF-I [38,39], these results have not been subsequently built on and they remain to be duplicated by other investigators. Whether WISP3 physically binds to IGF-I warrants further investigation, in light of our data.

Another mechanism that may explain the modulation of IGF-IR phosphorylation by WISP3-containing conditioned media is the formation of a WISP3/IGF-I complex that may bind to the IGF-IR and occupy IGF binding sites, but the complex may be either inhibitory or may be only a weak agonist of the receptor. In another system [40], this hypo-

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References


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