Overexpression of WISP-1 Down-regulated Motility and Invasion of Lung Cancer Cells through Inhibition of Rac Activation*

Lilian L. Soon‡, Ting-An Yie‡, Anita Shvarts‡, Arnold J. Levine§, Fei Su¶ and Kam-Meng Tchou-Wong**‡‡‡

From the ‡Division of Pulmonary and Critical Care Medicine, Departments of Medicine, **Environmental Medicine, and ¶Microbiology, New York University, New York, New York 10016, the ‡‡Institute for Advanced Study, School of Natural Science, Princeton University, New Jersey 08540, and ¶¶Hoffman-La Roche Inc., Nutley, New Jersey 07110

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Wnt-induced-secreted-protein-1 (WISP-1) is a cysteine-rich secreted protein that is induced by Wnt signaling and plays an important role in the regulation of cell motility and invasion. In this study, we investigated the effects of WISP-1 on the motility and invasion of lung cancer cells. We found that WISP-1 down-regulated Rac activation and inhibited the invasion of lung cancer cells. This effect was mediated through the inhibition of Rac activation, which is achieved by the interaction of WISP-1 with integrins. These findings have implications for the development of new therapeutic strategies for the treatment of lung cancer.
mice (10). WISP-1 also protected cells from p53-dependent apoptosis through activation of AKT, up-regulation of Bcl-XL, and inhibition of cytochrome c release (16).

While these proteins exert transforming and growth stimulatory effects on some cell types, they can also cause inhibition of growth and metastasis of tumor or transformed cells. The mouse equivalent of WISP-1, mELM1, was shown to be down-regulated in highly metastatic mouse melanoma cells. Consistently, upon mELM1 transfection and expression, the cells became less metastatic (17). In a differential-display study, the expression of rCOP-1, the rat equivalent of WISP-2, was significantly down-regulated in cells transformed by both activated H-Ras and an inactivated mutant of p53. When rCOP-1 was reintroduced into transformed cells, a high incidence of death occurred in the transfected cells (18). In Src-transformed chicken embryo fibroblasts, Nov was shown to be down-regulated in a transcriptional and also post-transcriptional manner (11). In addition, the expression of Nov was found to inhibit the growth of glioma cells (19). In non-small-cell lung cancer cells, CYR61 was shown to have tumor suppressor effects, and its expression was repressed in lung cancer samples compared with normal counterparts (20).

In the present paper, we showed that overexpression of WISP-1 in H460 cells reduced metastasis of these cells to the lungs in nude mice. In vitro studies demonstrated reduced invasion of WISP-1-expressing cells through Matrigel™ (BD Biosciences), a derivative of basement membranes. Metastasis and invasion require, in part, the migration of cells across the substratum. We showed that WISP-1-expressing cells were impaired in migration within Boyden chambers in response to serum. Biochemical pathways that regulate cell migration invoke the activation of small Rho-like GTPases, which function to reorganize cytoskeletal elements. WISP-1-expressing cells demonstrated marked inhibition of Rac activation. In the presence of blocking antibodies to integrins and WISP-1, Rac activation was restored. Transfection of a constitutively active Rac construct, RacG12V, into WISP-1 cells augmented the invasion and motility of these cells. Microarray analysis and real-time PCR showed that metalloproteinase-1 (MMP-1) expression was reduced in WISP-1 cells but was increased above control levels in RacG12V-transfected WISP-1 cells. These cells also showed increased invasion through collagen I, a substrate of MMP-1. These results were discussed in the context of WISP-1/integrin interactions and the effects on cell invasion and metastatic potential of lung cancer cells.

MATERIALS AND METHODS

Cell Culture and Transfections—H460 and H1299 cells as well as their transfecteds were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin, and streptomycin. H460 and H1299 cells were transfected with HA- and FLAG-tagged WISP-1 cDNA, and clonal cell lines were established as described previously (16). The constitutively active Rac mutant, RacG12V, was subcloned into the retrovirus vector pBabe and transfected into the packaging cell line, Phoenix A. The supernatant containing viable, non-replicative viruses was collected and used to infect H460-WISP1 cells. Stable cell populations were selected using puromycin. Control cell lines were also generated by infection with the pBabe empty vector. Conditioned medium was generated by incubating cells in serum-free medium. Three days later, the medium was collected, cleared of cell debris by centrifugation, concentrated 10-fold through a Centriplus YM-100 column (Millipore, Bedford, MA), and sterilized using a 0.45-μm polyvinylidene difluoride filter (Whatman, Clifton, NJ) before use.

Western Blotting and Immunoprecipitation—Cells were trypsinized and counted, and 3 x 10^6 cells were seeded in 10-cm dishes for 24 h. The cells were then starved in serum-free DMEM for 16 h followed by induction with serum for various periods of time. Serum stimulation was stopped by the addition of cold PBS, and the cells were lysed in RIPA buffer containing 1% SDS and 1% deoxycholate. Protein assay was used to determine equivalent amounts of protein for gel loading, and the lysate was added to sample buffer and boiled for 8 min. Proteins were fractionated by electrophoresis on SDS gels and transferred onto polyvinylidene difluoride immobilization membranes. The membranes were blocked with 5% milk and incubated with primary antibodies followed by washes and staining with secondary antibodies. Detection and visualization was by chemiluminescence (PerkinElmer Life Sciences) followed by exposure to X-OMAT film (Kodak, Rochester, NY).

In Vivo Lung Metastasis Assay—An in vivo lung metastasis experiment, vector control or WISP-1-expressing H460 cells (10^6 cells/0.1 ml of DPBS) were injected into the lateral tail veins of nude mice (Taconic, Germantown, NY). Eight weeks later, the animals were sacrificed, and the lungs were fixed in formalin. Lung sections were processed, stained with hematoxylin and eosin, and observed for tumor cell colony formation.

Migration and Invasion Assays—Cells were trypsinized and counted, and 2 x 10^6 cells were placed in upper migration chambers (BD Biosciences), which were then rested in wells containing 1.5% FBS in DMEM. Incubation was carried out for 8 h in a humidified CO_2 chamber. Non-migratory cells in the upper chamber were removed by scraping, and migrated cells on the lower surface were fixed in methanol and stained with methylene blue. The number of migrated cells were then counted from a total of nine regions of the filter and calculated as numbers/cm^2. For the invasion assays, 4 x 10^6 cells were placed in upper chambers precoated with a layer of Matrigel with 5% FBS present in the lower chamber. The cells were incubated for 48 h and then processed as described above. Collagen I-coated wells were made by adding 2 μg/ml of collagen I (Chemicon, Temecula, CA) to 19 μg in upper chambers and drying for a few hours at room temperature. Cell invasion assays in collagen I-coated filters were performed as described above.

Rac Activation Assay—Equal numbers of cells were seeded and incubated for 24 h in a 5% CO_2 chamber, serum-starved for 16 h, and treated with DMEM containing 10% FBS or no serum for 15 min. The reaction was stopped with cold DPBS, the cells were lysed with lysis buffer containing Mg^{2+}, and the lysate was centrifuged for 10 min. The supernatant (0.5 μg of proteins) was added to 10 μl of p21-activated kinase (PAK)-agarose conjugates (UBI, Lake Placid, NY), rotated at 4 °C for 75 min, and followed by three washes of the protein complex with lysis buffer. PAK-bound proteins were dissociated and denatured by heating in sample buffer at 98 °C for 8 min and subjected to gel electrophoresis. Rac proteins were visualized using anti-Rac antibody (UBI) and chemiluminescence techniques. Inhibitory antibodies to the integrins α5, α6β4, and αv (Chemicon) were added to cell cultures 24 h prior to treatment with serum and harvesting for activation assays.

Microarray Analysis and Real-time PCR—H460, WISP-1, and H460/WISP-1/RacG12V cells were seeded to equal density and incubated for 24 h in a 5% CO2 chamber. The cells were washed twice with DPBS and lysed for RNA precipitation using RNeasy mini-columns (Qiagen, Valencia, CA). The RNA was used to generate double-stranded cDNA (Invitrogen), which was then transcribed into biotinylated cRNA (Affymetrix, Santa Clara, CA). The fragmented products were hybridized to U133A gene chips and processed in the fluidics station (Affymetrix). The probe array was scanned, and the data analyzed using the Microarray Suite Software (Affymetrix).

Real-time PCR reactions involved first-strand synthesis from RNA by oligo(dT) priming (Ambion, Austin, TX), addition of template to a PCR mix containing the SYBR® green reporter molecule (Applied Biosystems, Foster City, CA), and running the PCR reaction in the ABI Prism 7000 Sequence Detector (Applied Biosystems). The comparative cycle threshold (Ct) method was used to analyze the data by generating relative quantities of the amount of target cDNA, p21-activated, by heating in sample buffer at 98 °C for 8 min and subjected to gel electrophoresis. The calculated result represents the amount of normalized target relative to the calibrator.

RESULTS

WISP-1 Expression Inhibits Metastasis and Invasion of H460 Cells—The large-cell lung cancer line, H460, exhibits properties of neuroendocrine cells and is highly metastatic. To investigate the role of WISP-1 in metastasis, H460 cells transfected with the human WISP-1 gene or vector control were injected into the tail vein of nude mice and monitored for tumor growth.
Overexpression of WISP-1

in vitro invasion of H460 lung cancer cells. A, H460/vector control formed large and extensive colonies of tumors in the lungs of nude mice. In contrast, tumor size was reduced in WISP-1 expressors. Arrows indicate tumor masses, which stained darker than the normal lung parenchyma. B, in an in vitro invasion assay, H460/WISP-1 cells demonstrated reduced invasiveness across Matrigel compared with control cells. C, H460 cells were less invasive in the presence of WISP-1-conditioned medium (WISP-1CM) compared with vector control conditioned medium (vectorCM).

Overexpression of WISP-1 inhibits in vivo metastasis and in vitro invasion of H460 lung cancer cells. A, H460/vector control formed large and extensive colonies of tumors in the lungs of nude mice. In contrast, tumor size was reduced in WISP-1 expressors. Arrows indicate tumor masses, which stained darker than the normal lung parenchyma. B, in an in vitro invasion assay, H460/WISP-1 cells demonstrated reduced invasiveness across Matrigel compared with control cells. C, H460 cells were less invasive in the presence of WISP-1-conditioned medium (WISP-1CM) compared with vector control conditioned medium (vectorCM).

in the lungs. Eight weeks after injection, vector-transfected cells were found to be metastatic to the lung and formed large tumor clusters, whereas WISP-1-expressing cells had markedly reduced numbers and size of tumor colonies, indicating that WISP-1 expression inhibited lung metastasis (Fig. 1A). H460 transfectants were also used in an in vitro assay for invasion using Matrigel-coated chambers. WISP-1-expressing cells demonstrated considerably reduced invasiveness across Matrigel compared with vector control cells (Fig. 1B). An invasion assay using conditioned medium generated from either H460 vector control (vectorCM) or WISP-1-expressing cells (WISP-1CM) was conducted (Fig. 1C). The invasion of H460 cells incubated with vectorCM was significantly greater than that with WISP-1CM, indicating inhibition of invasion by soluble WISP-1 secreted into the conditioned medium (Fig. 1C). Data represent the mean ± S.D. of triplicate determinations.

WISP-1 Down-regulates the Motility of Lung Cancer Cells in Boyden Chamber Assays—The motility of cells incorporates changes in the cytoskeleton that affect cellular polarization and adhesion. A component of the process of invasion is the motility of cells across a substratum. Since WISP-1 in H460 cells inhibited metastasis and invasion, we tested whether the expression of WISP-1 may reduce the motility of cells in vitro using Boyden chamber assays. A total of 2 × 10⁴ cells in serum-free medium were placed in the upper Boyden Chamber and induced to move across a gradient of serum factors (1.5% FBS). Following incubation for 8 h in a 5% CO₂ incubator, H460/WISP-1 cells were significantly less motile compared with vector transfectants. A checkerboard analysis was performed that confirmed that H460 cells preferentially migrated across a gradient of chemotactic serum factors (data not shown). Similar inhibition by WISP-1 in the motility of H1299 lung cancer cells was also observed (Fig. 2).

Rac Activation Is Inhibited in WISP-1-expressing Cells—Cell migration is regulated by pathways that involve cytoskeletal rearrangements resulting in membrane ruffling, lamellae formation, and cell protrusions. These cellular activities are controlled by Rho-like GTPases such as Rho, Rac, and Cdc42. To investigate whether functional changes in cell motility by WISP-1 expression in lung cancer cells may be related to the activity of Rho GTPases, WISP-1-expressing and vector control cells were subjected to an indirect assay for Rac activation. The results showed that even though equivalent amounts of total Rac protein were present, WISP-1-expressing cells exhibited reduced binding of GTP-bound Rac (activated Rac) to PAK/agarose conjugates compared with control cells. Similar results were obtained for WISP-1-expressing H1299 cells that exhibited diminished precipitation of activated Rac compared with control cells, indicating a reduction in Rac activity (Fig. 3).

Restoration of Rac Activation in the Presence of Inhibitory Antibodies—CCN family members have been shown to bind and activate integrins. To determine whether the inactivation of Rac by WISP-1 may involve upstream components such as integrins, inhibitory antibodies to integrins α₁, caused an increase in Rac activation in WISP-1-expressing cells but not control cells (Fig. 4A). When α₁ β₃ and α₁ antibodies were added together, the effect on the activation assay was greater than either antibodies alone (Fig. 4A). On the other hand, there was no effect of these antibodies on vector-expressing cells (Fig. 4B).

To further ascertain the role of WISP-1 in the inhibition of Rac activation, affinity-purified antibodies previously shown to have a blocking effect on WISP-1 (16) were used in a Rac activation assay. WISP-1 blocking antibodies inhibited the effect of WISP-1 on Rac and restored Rac activation, indicating specificity of function of WISP-1 (Fig. 4B).
Rac activation in WISP-1 cells. Antibodies to integrins. Blocking antibodies to WISP-1 also reversed the inhibition of Rac activation by WISP-1.

Microarray studies and bioassays.

Metalloproteinase-1 is down-regulated by WISP-1 but up-regulated by RacG12V—The inhibition of cell invasion by WISP-1 may involve changes in the expression of genes that regulate matrix turnover or degradation. To determine how the expression of WISP-1 may alter the expression of genes involved in cellular invasion, microarray analysis was performed using cRNA derived from H460/vector, H460/WISP-1, and H460/WISP-1/RacG12V transfectants. The biotin-labeled cRNA was hybridized to gene chips, which were then processed and scanned. The data obtained were used in binary comparative analyses using the Microarray Suite Software. Genes significantly different in expression were identified based on calculated p values. Among the genes of interest, MMP-1 was identified as a gene down-regulated in WISP-1-expressing cells but up-regulated in the WISP-1/RacG12V cells, which may play role in cell invasion.

To verify this data, real-time PCR was conducted using primers specific to MMP-1 and actin. Target amounts were normalized against actin, and the H460 control target was used as a calibrator (see “Materials and Methods”). As shown in Fig. 6, WISP-1 cells showed ~3-fold lower expression of MMP-1 than control cells. WISP-1/RacG12V cells, on the other hand, had increased values of about 3-fold higher than control cells. These results indicated that WISP-1 inhibited the expression of MMP-1, whereas activation of Rac restored the level of MMP-1 in WISP-1-expressing cells (Fig. 6).

Migration and Invasion of RacG12V-transfected WISP-1-expressing Cells—To test whether increased Rac activity in WISP-1/RacG12V cells may restore motility and invasion in these cells, a modified Boyden Chamber assay was conducted. The assay showed that reconstitution of Rac activity in H460/WISP-1/RacG12V cells increased the motility of these cells compared with WISP-1 cells (Fig. 7A). Similarly, WISP-1/RacG12V cells also exhibited higher levels of invasion than WISP-1 cells, indicating that activation of Rac promotes cellular invasion in vitro (Fig. 7B). MMP-1 proteolyses several types of substrate including collagen type I. A collagen I invasion assay was used to determine whether reduced expression of MMP-1 by WISP-1 may affect the invasion of cells across a target substrate. The upper wells of migration chambers were coated with 19 μg of collagen I, and the lower chambers were filled with 5% FBS-containing medium. Consistent with the reduced expression of MMP-1 (Fig. 6), WISP-1 cells were impaired in the invasion of collagen I compared with control cells. WISP-1/RacG12V cells were, however, as efficient as control cells in the invasion assay (Fig. 7C), which correlated with increased MMP-1 expression by RacG12V transfection.

DISCUSSION

Members of the CCN family play important roles in development during chondrogenesis, skeletogenesis, and neurogenesis. In adults, they may serve to maintain matrix structure and composition and tissue homeostasis. How the natural functions of CCN proteins relate to cancer and other diseases is not well understood. In addition, the correlation between the incidence and progression of cancer with the expression of CCN proteins cannot be generalized across different types of cancer. This may be due to the presence of distinct cohorts of receptors and/or variable regulation of the ligands at the cell surface, such as cleavage by proteases, in a cell type-specific manner. In addition, the response of CCN proteins to a given environment is strongly influenced by its multidomain structure, which may
The mechanism of cell invasion involve both the secretion metalloproteinases, which digest basement membrane (24), and the motility of cells. Motile cells adopt a polar morphology in the form of distinctive front and trailing edges. The motility of cells also require the cycling of localized attachment and detachment from the surface of matrices. The Rho family of GTPases has been characterized for their involvement in cellular actin reorganizations during the motility of cells (25). Small GTPases cycle between an active and inactive state through the hydrolysis of GTP. Cdc42 regulates pseudopod extensions, whereas Rac and Rho initiate the formation of lamellae and the establishment of stress fibers, respectively. The activities of Cdc42 and Rac propel cell motion by polarizing cells into a motile phenotype. Rho, on the other hand, generally, stabilized stress fibers and focal adhesions, thereby inhibiting cell motility (25).

In this paper, we studied the effects of WISP-1, a Wnt-1-inducible factor belonging to the CCN family, on the metastasis and invasion of lung cancer cells. When H460 and H1299 lung cancer cells were transfected with WISP-1, lamellar structures were largely reduced, and the cells were less spread on the surface of tissue culture plates (16). In vitro motility and invasion of WISP-1-expressing H460 and H1299 as well as the in vivo metastasis of H460/WISP-1 cells were significantly reduced compared with vector control cells. These results prompted the hypothesis that the activation of Rac may be inhibited by WISP-1, resulting in the reduction of lamellar structures, cell motility, and invasion.

In an indirect assay for Rac activation, we observed a marked reduction in the activation of Rac for WISP-1 transfectants of both H460 and H1299 cells compared with control cells. Upon transfection of a constitutively active Rac mutant, RacG12V, increases in the motility and invasion of cells were observed for H460/WISP-1/RacG12V cells, demonstrating that the down-regulation of Rac activity by WISP-1 was responsible for the inhibition of cell motility and invasion.

The activation of Rac occurs downstream of several pathways including integrin/Src kinase (26) and platelet-derived growth factor signaling (27). In addition, the pertussis toxin-sensitive G protein, Gi (28), and cAMP-dependent pathways (29) have also been implicated in Rac-mediated cell spreading and migration. To understand how WISP-1 may be inhibiting Rac, a number of inhibitors were used in the Rac activation assay. We found that none of the inhibitors to phosphatidylinositol 3-kinase, Src, G-proteins, and calmodulin were able to inhibit endogenous Rac activity significantly in control cells (data not shown). On the other hand, when inhibitory antibodies to several integrins were used, Rac activation in WISP-1 cells was restored to almost wild-type levels, while there was no effect on control cells. Similar observations were made using blocking antibodies to WISP-1. Therefore, the down-regulation of Rac activation by WISP-1 may be responsible for the inhibition of cell motility and invasion.

In addition, a consequence of WISP-1 overexpression is the down-regulation of MMP-1, a metalloproteinase involved in lung cancer metastasis, which may occur through WISP-1 inhibition of Rac activation. Consistently, the invasion of cells across collagen I, a substrate of MMP-1, was compromised in WISP-1-transfected cells but was increased in the WISP-1/RacG12V transfectants.

It is unclear how WISP-1 may regulate Rac function through integrins. The mechanism may involve a shift in the balance of regulatory factors, such as bombesin (gastrin-releasing peptide), and stimulation of endogenous receptors (23).
activatory and inhibitory signals. Mechanosensory effects mediated through integrins may produce cytoskeletal-mediated signaling operating through actin-myosin and microtubular dynamics (30). However, it is possible that the inhibition may occur through changes in activity of Rac regulators. As an example, mechanical stresses applied to fibroblasts resulted in the inhibition of Rac activity and lamellipodia formation, with implications that a guanine exchange factor may be responsible (31). Therefore, the effect of WISP-1 on integrin signaling may be multiterritorial. On one level, the activating functions of integrins may be altered or absent. On another level, a set of alternative reactions may occur, triggered by cell shape and cytoskeletal changes.

Integrins are the only known receptors for CCN proteins, and receptor activation may produce a variety of effects. For example, interaction of CYR61 with integrin αvβ3 promotes cell adhesion and chemotaxis in vascular smooth muscle cells (32). In human skin fibroblasts, CYR61 stimulates migration and proliferation through integrin αvβ3 and integrin αvβ5, respectively (33). In contrast, when CYR61 was overexpressed in lung cancer cells, tumor growth was suppressed (20).

Similarly, WISP-1 was reported to have tumorigenic properties when expressed in normal rat kidney fibroblasts (7), whereas in melanoma cells, contrasting effects were observed and WISP-1-expression resulted in the inhibition of tumorigenesis and metastasis. Consistent with the latter, we demonstrated that expression of WISP-1 in H460 lung cancer cells inhibited lung metastasis in mammary cancer tissue from Wnt-1 transgenic mice, mesenchymal cells demonstrated high levels of expression of WISP-1, whereas in cancer cells, low levels or negative staining was observed. This suggests that the secretion of WISP-1 by stromal cells may have positive regulatory effects on cancer cells through paracrine signaling (5). On the other hand, autocrine WISP-1 production by cancer cells may result in the inhibition of tumorigenesis and metastasis, and therefore, its expression may be down-regulated in cancer. It is plausible that there may be differences between cancer and normal cells in the processing and presentation of WISP-1 on the cell surface. This in turn may cause variability in WISP-1 interactions with integrins that result in specific signaling and biological outcomes in their respective cell types. Some support for this comes from our unpublished data showing that the secreted form of WISP-1 from H460 lung cancer cells is highly glycosylated and that the pattern of glycosylation differs between cancer cells and normal fibroblasts. Investigations are being carried out to evaluate the functional significance of the data. Finally, further studies will be needed to determine if the expression or processing of WISP-1 in tumors may have prognostic value in predicting cancer outcome.

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