Wild-Type p53 Attenuates Cancer Cell Motility by Inducing Growth Differentiation Factor-15 Expression

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A major function of the p53 tumor suppressor is the regulation of the cell cycle and apoptosis. In addition to its well-documented functions in malignant cancer cells, p53 can also regulate cell migration and invasion, which contribute to metastasis. Growth differentiation factor-15 (GDF-15), a member of the TGF-β superfamily, has been shown to be a downstream target of p53 and is associated with diverse human diseases and cancer progression. In this study, we examined the potential role of GDF-15 in p53-regulated cancer cell motility. We show that overexpression of wild-type p53 in two highly invasive p53-null human cancer cell lines, SKOV3 and PC3, attenuated cell migration and the movement through Matrigel. Using wild-type p53 and DNA-binding-deficient p53 mutants, we found that the transcriptional activity of p53 is required in the induction of GDF-15 expression. Cell movement through uncoated and Matrigel-coated transwell decreased in response to treatment with recombinant GDF-15, whereas the cell proliferation was not affected by GDF-15 treatment. Moreover, the induction of GDF-15 expression and secretion by p53 and the reduction in cell movement through Matrigel were diminished by treatment with GDF-15 small interfering RNA. This study demonstrates a mechanism by which p53 attenuates cancer cell motility through GDF-15 expression. In addition, our results indicate that GDF-15 mediates the functions of p53 by autocrine/paracrine action. (Endocrinology 152: 2987–2995, 2011)

Cancer is a leading cause of death worldwide. Cancer cells are defined by two defining properties: uncontrolled cell growth and invasion (1). Mutations in cell cycle regulatory genes mainly contribute to the loss of control of cell growth and the formation of a primary tumor. During tumorigenesis, the most critical step is the progression of a static primary tumor into an invasive tumor because the spread of cancer cells to other locations in the body via lymph or blood significantly decreases the efficiency of treatment and prognosis. p53 was first discovered in 1979 and was found to be in a complex with the SV40 (simian virus 40) antigen (2, 3). Subsequent studies revealed that wild-type p53 acts as a tumor suppressor (4) and is frequently mutated in diverse types of human cancer (5). Recently, reactivation of wild-type p53 in tumor cells has been proposed as a potential therapy for human cancer (6). An in vivo study showed that transplanting breast cancer cells into p53-null mice reduced the latency for tumor development compared with wild-type mice (7). p53 has been extensively studied for its role in the control of cell cycle, apoptosis, and DNA repair; more recent studies have suggested that p53 is responsible for other cellular functions, such as cell adhesion, migration, invasion, and cytoskeletal organization (8).

Growth differentiation factor-15 (GDF-15) is a divergent member of the TGF-β superfamily (9). Aliases for GDF-15 are macrophage inhibitory cytokine-1, non-steroidal antiinflammatory drug-activated gene-1, prostate-derived factor, placental TGF-β, and placental bone morphogenetic protein (10). In normal physiological conditions, the placenta is the only tissue that expresses high levels of GDF-15 (11). GDF-15 expression increases dramatically in conditions of cellular stress, acute injury, inflammation, and cancer (12, 13). In ovarian, prostate, and...
colon cancers, serum GDF-15 levels increase significantly with disease progression and may be a potential biomarker for those cancers (14–16). To date, GDF-15 receptors have not been well defined. However, studies have suggested that GDF-15 can regulate a wide variety of cellular processes, including growth inhibition, induction of apoptosis, and tumor invasiveness (10).

The GDF-15 gene promoter contains two consensus p53 binding sites, and wild-type p53 can induce GDF-15 expression in both in vitro and in vivo models (17, 18). In addition, other transcription factors, such as Egr-1, nuclear factor-κB, and hypoxia-inducible factor-1α, have been shown to induce GDF-15 expression in different cell types (10). The functional connection between p53 activity and cell motility has been established by studies concerning the influence of p53 on Rho GTPases. One study showed that Rho family GTPases cooperate with p53 deletion to promote primary mouse embryonic fibroblast cell invasion (19). However, whether GDF-15 mediates wild-type p53-induced attenuation of cancer cell migration and invasion is currently unknown.

In this study, we show that overexpression of wild-type p53 in two invasive p53-null cancer lines, SKOV3 and PC3, increased GDF-15 expression and attenuated cell motility. In addition, treatment with recombinant human GDF-15 decreased the cell movement through uncoated and Matrigel-coated transwell. Moreover, the induction of GDF-15 by p53 and the reduction in cell movement through Matrigel were diminished by treatment with GDF-15 small interfering RNA (siRNA). Our results demonstrate an additional mechanism of the p53/GDF-15 pathway by which it regulates tumor cell motility.

Materials and Methods

Cell culture

The human ovarian cancer cell line SKOV3 and human prostate cancer cell line PC3 were obtained from American Type Culture Collection (Manassas, VA). SKOV3 cells were grown in a 1:1 (vol/vol) mixture of M199/MCDB105 medium (Sigma-Aldrich, Oakville, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT). PC3 cells were maintained in RPMI 1640 medium (Invitrogen, Burlington, Ontario, Canada) with 10% FBS. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 in air.

Antibodies and reagents

A polyclonal anti-GDF-15 antibody (no. 3209) was purchased from Cell Signaling Technology (Danvers, MA). Polyclonal anti-p53 (sc-6243) and polyclonal anti-β-actin (sc-1615) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG was obtained from Bio-Rad Laboratories (Hercules, CA). Horseradish peroxidase-conjugated donkey antigoat IgG was obtained from Santa Cruz Biotechnology. Recombinant human GDF-15 was obtained from R&D Systems (Minneapolis, MN).

Transwell assays

Transwell assays were performed in Boyden chambers with minor modifications (20). Cell culture inserts (24-well, pore size 8 μm; BD Biosciences, Mississauga, Ontario, Canada) were seeded with 1 × 105 cells in 250 μl medium with 0.1% FBS. Uncoated inserts or inserts precoated with growth factor-reduced Matrigel (40 μl, 1 mg/ml; BD Biosciences) were used. Medium with 10% FBS (750 μl) was added to the lower chamber and served as a chemotactic agent. After 12 h (uncoated) or 48 h (Matrigel-coated) incubation, nonmigrating/invasive cells were wiped from the upper side of the membrane, and cells on the lower side were fixed in cold methanol (−20°C) and air dried. Cell nuclei were stained with Hoechst 33258 and counted using a Zeiss Axiohot epifluorescent microscope (×10 objective) equipped with a digital camera (Qimaging, Surrey, British Columbia, Canada). Each individual experiment had triplicate inserts and five microscopic fields (obtained from middle, upper, lower, right, and left parts of membrane) were counted per insert by Northern Eclipse version 6.0 software. The quantification was performed with the operator blinded to the treatment groups.

MTT assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) was used to determine cell viability. Cells were transfected with either empty vector or pCMV-p53 for 24 h and then seeded in a 24-well plate (1 × 104 per well) with 500 μl medium. MTT was added at different time points to a final concentration of 0.5 mg/ml and then incubated for 4 h. The medium was removed, and dimethylsulfoxide was added into each well to dissolve the crystals. The absorbance values were examined by spectrophotometer microplate reader.

siRNA transfection and protein overexpression

To knock down GDF-15, the cells were transfected with ON-TARGETplus SMARTpool GDF-15 (50 nM) siRNA (Dharmacon Research, Inc., Lafayette, CO) using Lipofectamine RNAiMAX (Invitrogen). The siCONTROL NON-TARGETING pool siRNA (Dharmacon) was used as the transfection control. For protein overexpression, 1 μg pCMV vector encoding a human wild-type or deficient in DNA binding p53 mutants (V143A, R175H, R248W, R273H) (21) (Addgene, Cambridge, MA) was transfected into cells using Lipofectamine 2000.

Western blot analysis

Cells were lysed in cell lysis buffer (Cell Signaling). Equal amounts of protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. After blocking with Tris-buffered saline containing 5% nonfat dry milk for 1 h, membranes were incubated overnight at 4°C with GDF-15 (1:1000) and p53 (1:1000) primary antibodies diluted in 3% BSA/Tris-buffered saline, followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:5000). Immunoreactive bands were detected with an enhanced chemiluminescent substrate. Membranes were stripped with stripping buffer at 50°C for 30 min and reprobed with anti-β-actin (1:5000) as a loading control.
RT-quantitative real-time PCR (RT-qPCR)

Total RNA was extracted using TRizol reagent (Invitrogen), according to the manufacturer’s instructions. RT was performed with 3 μg RNA, random primers, and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). The primers used for SYBR Green RT-qPCR were as follows: GDF-15, 5′-CTC CAG ATT CCG AGA GTT GC-3′ (sense) and 5′-AGA GAT ACG CAG GTG CAG GT-3′ (antisense); and GAPDH, 5′-GAG TCA ACG GAT TTG GTG GT-3′ (sense) and 5′-GAC AAG CTT CCC GTT CTC AG-3′ (antisense). RT-qPCR was performed on an Applied Biosystems 7300 Real-Time PCR System (PerkinElmer, Norwalk, CT) equipped with a 96-well optical reaction plate. All RT-qPCR experiments were run in triplicate, and a mean value was used for the determination of mRNA levels. Relative quantification of the mRNA levels was performed using the comparative cycle threshold (Ct) method with GAPDH as the reference gene and with the formula 2^{ΔΔCt}.

GDF-15 ELISA

A human GDF-15-specific ELISA was used in accordance with the manufacturer protocol (R&D Systems). Briefly, cells were transfected with empty vector or vector encoding wild-type p53 in combination with control siRNA or GDF-15 siRNA for 48 h. The culture media were collected, and the GDF-15 levels in the culture media were measured by ELISA. GDF-15 levels were normalized to the protein concentrations from the cell lysates. Normalized GDF-15 values from the treatments were represented as relative values by comparing with control treatment.

Statistical analysis

Results

Overexpression of wild-type p53 decreases cell motility in SKOV3 and PC3 cells

To examine the effect of reactivation of wild-type p53 on cancer cell motility, we chose two invasive p53-null cancer cell lines, the human ovarian cancer cell line SKOV3 and the prostate cancer cell line PC3, as our in vitro models. To overexpress wild-type p53, a pCMV vector encoding human wild-type p53 was transfected into these two cell lines, and the expression levels of p53 were confirmed by Western blot analysis (Fig. 1A). As shown in Fig. 1B, the overexpression of wild-type p53 significantly attenuated cell migration through uncoated transwell in both SKOV3 and PC3 cells. In addition, the overexpression of wild-type p53 also attenuated cell movement through Matrigel in both SKOV3 and PC3 cells (Fig. 1C). p53 can regulate cell cycle progression and promote apoptosis. To confirm that the inhibitory effect of wild-type p53 on cell motility was not due to differences in cell growth, cell proliferation after p53 transfection was examined using a MTT assay. In both SKOV3 and PC3 cells,
wild-type p53 slightly decreased cell proliferation after 4 d of culture, but the difference was not statistically significant (Fig. 1B).

Overexpression of wild-type p53 increases GDF-15 expression in SKOV3 and PC3 cells

To determine whether p53 induces GDF-15 expression in our model system, the mRNA levels of GDF-15 were examined in SKOV3 and PC3 cells overexpressing wild-type p53. RT-qPCR analysis showed that overexpression of p53 increased GDF-15 mRNA levels in a time-dependent manner (Fig. 2A). p53 modulates cellular responses by directly inducing gene transcription or by interacting with other proteins (22). To investigate whether p53-induced GDF-15 expression occurs through a transcription-dependent mechanism, SKOV3 and PC3 cells were transfected with either wild-type p53 or different p53 mutants, in which the DNA-binding domain was mutated (21). RT-qPCR analysis showed that GDF-15 mRNA levels were significantly induced by wild-type p53 but not by p53 mutants (V143A, R175H, R248W, and R273H) (Fig. 2B).

Western blot analysis showed that the baseline levels of GDF-15 are very low in both SKOV3 and PC3 cells. Similarly, GDF-15 protein levels were increased by wild-type p53 but not by p53 mutants (Fig. 2C). These results suggest that the transcriptional activity of p53 is required for the p53-induced GDF-15 expression.

Treatment of GDF-15 decreases SKOV3 and PC3 cell motility

GDF-15 has been shown to influence cell migration and invasion in a manner dependent on cell type and context (10). To determine the effect of GDF-15 treatment on cell motility, SKOV3 and PC3 cells were treated with different doses of human recombinant GDF-15 (20, 50, and 100 ng/ml), and then cell movement through uncoated and Matrigel-coated transwell was examined. As shown in Fig. 3, A and B, GDF-15 treatment significantly decreased cell movement through uncoated and Matrigel-coated transwell in a dose-dependent manner in both SKOV3 and PC3 cells. In addition, treatment with GDF-15 did not alter the cell proliferation after 4 d of culture (Fig. 3C). These results indicate that the inhibitory effects of wild-type p53 on cell motility were not due to differences in cell growth.

Autocrine/paracrine action of GDF-15 is required for p53-induced attenuation of SKOV3 and PC3 cell motility

To examine whether GDF-15 is required for p53-dependent decreased cell motility, specific siRNA for GDF-15 was used to knock down the endogenous and p53-induced GDF-15. RT-qPCR analysis showed that GDF-15 siRNA not only blocked the p53-induced GDF-15 mRNA levels but also decreased the endogenous GDF-15 mRNA levels in both SKOV3 and PC3 cells (Fig. 4A). Similarly, changes in GDF-15 protein levels were shown by Western blot analysis (Fig. 4B). It has been shown that GDF-15 is a secreted protein (9). To examine whether p53 can induce GDF-15 secretion, the levels of GDF-15 in the culture media were measured by ELISA. As shown in Fig. 4C, overexpression of wild-type p53 increased GDF-15 secretion. In addition, GDF-15 siRNA not only blocked the p53-induced GDF-15 secretion but also decreased the basal levels of GDF-15 secretion in both SKOV3 and PC3 cells. Furthermore, the inhibitory effects of overexpressing wild-type p53 on cell movement through Matrigel-coated transwell were diminished by cotreatment with GDF-15 siRNA (Fig. 5).
Taken together, these results indicate that the induction and autocrine/paracrine action of GDF-15 are required for p53-induced inhibition of cell motility.

Discussion

The tumor suppressor p53 is frequently mutated in many types of human cancer, and the p53 pathway now is a major target in the development of new cancer drugs and therapies (23). Therefore, a better understanding of the molecular mechanisms of p53 function will benefit the development of optimal treatment strategies. In the present study, we demonstrated that the overexpression of wild-type p53 in two highly invasive p53-null human cancer cell lines attenuated cell movement through uncoated and Matrigel-coated transwell, and this process required p53-induced GDF-15 expression and secretion.

Cell migration and invasion are implicated in the pathophysiology of cancer. It has been shown that p53 can regulate cell migration and invasion by regulating the cytoskeleton remodeling and matrix metalloproteinases (MMP) (24, 25). To date, only a few studies have investigated the function of GDF-15 in cell motility, and its role in cell motility is still ambiguous. GDF-15 overexpression has been shown to enhance cell migratory and invasive abilities by the activation of focal adhesion kinase-RhoA signaling pathway-mediated actin reorganization (26). One study showed that a higher expression level of GDF-15 correlated with a more invasive phenotype in human gastric cancer cell lines (27). Moreover, overexpression of GDF-15, or direct treatment with recombinant GDF-15, induced gastric cancer cell invasion by up-regulating the urokinase-type plasminogen activator system (27). GDF-15 strongly inhibited human umbilical vein endothelial cell migration and invasion through Matrigel (28). The differences in the signaling molecules involved in the mediation of GDF-15 have been suggested to influence the final cellular response induced by GDF-15 in a given cell type (10). In this study, we showed that treatment with recombinant GDF-15 decreased cell movement through uncoated and Matrigel-coated transwell in human ovarian...
and prostate cancer cell. Moreover, p53-induced attenuation of cell movement through Matrigel was diminished by treatment with GDF-15 siRNA. However, GDF-15 siRNA only partially diminished the inhibitory effect of p53 on cell movement through Matrigel, suggesting the presence of other molecules that are involved in p53/GDF-15-mediated attenuation of cell motility.

Cell invasion is similar to cell migration; however, it requires a cell to migrate through an extracellular matrix or basement membrane barrier by enzymatically degrad- ing the barrier. Matrigel-based invasion assays have been used extensively to monitor the invasive potential of a variety of cancer cell types including ovarian and prostate cancer cells (27, 29). However, it has been shown that amoeoboid tumor cell motility can occur even after inhibition of extracellular proteases, including MMP (30, 31). In ovarian cancer, the broad range MMP inhibitor completely prevents cell perforation of polymerized collagen I-coated transwell membranes, whereas it does not or only partially blocks the Matrigel penetration in different types of ovarian cancer cells (32). In the present study, our results showed that overexpression of p53 or GDF-15 treatment attenuated cell movement through uncoated and Matrigel-coated transwell. These results indicated that overexpression of p53 and GDF-15 treatment can attenuate cell motility. However, the requirement of MMP for the cells to penetrate the Matrigel is unknown. We are aware of this, and hence, future studies will be required to address the question of whether wild-type p53 or GDF-15 can regulate protease-dependent cell invasion in SKOV3 and PC3 cells.

Numerous in vitro and in vivo studies have revealed that GDF-15 can induce various pleiotropic effects during cancer progression by negatively or positively modulating cell proliferation, apoptosis, migration, and invasion in a manner dependent on cancer cell types, disease stage, and tumor microenvironment (10). In nonneoplastic tissue, GDF-15 has been shown to regulate cartilage and endochondral bone formation, adipose tissue function, and cardiac hypertrophy and promote fetal survival by suppressing the production of maternally derived proinflammatory cytokines within the uterus (10). In the malignant cells, GDF-15 can display its antitumorigenic properties by regulating cell proliferation and apoptosis (17, 33). In contrast, secreted GDF-15 may contribute to the acquisition of a more malignant behavior and enhanced invasive and metastatic abilities in cancer cells (27, 34). In the present study, we found treatment with recombinant GDF-15 attenuated ovarian and prostate cancer cell motility but did not affect the cell proliferation. These results suggest that the increased levels of GDF-15 may have a homeostatic role and act as a metastasis suppressor during the cancer progression. However, the inhibitory effect of GDF-15 on the cell motility is opposite to the previous studies (27). In this regard, these results may suggest that the genetic and/or epigenetic alterations in the signaling elements involved in the mediation of GDF-15 effects on cancer cells or modulators of these pathways may occur in the different types of cancer cells and thereby influence the final cellular response induced by GDF-15 in a given cell type. Hence, whether GDF-15 has the same inhibitory effects on cell motility in other types of cancer cells will need further investigation.

Autocrine and paracrine effects are important mechanisms that mediate the biological functions of TGF-β superfamily members (35). GDF-15 is a secretory protein, and elevated serum GDF-15 levels are associated with a number of disease conditions. GDF-15 level is usually low in resting cells but may be substantially
increased after an adaptive response to diverse cellular stress signals, such as hypoxia, inflammation, and acute tissue injuries, and during cancer progression (10). In nonmalignant tissue, it has been shown that GDF-15 concentration in serum increases dramatically during pregnancy, which in the first trimester contains approximately 6.3 ng/ml GDF-15, rises to 12.2 ng/ml during the second trimester, and peaks at 15.3 ng/ml during the third trimester (36). In human ovarian and prostate cancers, serum GDF-15 levels increase significantly with disease progression and may be deemed as a potential biomarker (14, 16). The serum GDF-15 concentrations in the ovarian and prostate patients are ranged from 1–12.5 ng/ml (13, 14, 37). Our results showed that 50 and 100 ng/ml recombinant GDF-15 treatment significantly attenuated cell motility. These doses are higher than the level of GDF-15 in the serum from either normal people or cancer patients. However, the exact concentration of GDF-15 in the cancer microenvironment is unknown. To date, the lack of precise information on the GDF-15 receptor and other regulatory factors of signal transduction pathways induced by this cytokine underline the urgent need to further establish their identities and implications in mediating the specific cellular responses induced by secreted GDF-15 in a given cell type. Thus, future studies are required to determine the potential implications of GDF-15 that may help researchers to develop new therapeutic approaches against a variety of human pathological disorders and cancers.

In the present study, we showed that GDF-15 siRNA not only blocked p53-induced GDF-15 mRNA levels but also decreased endogenous GDF-15 mRNA levels. In addition, the ELISA results showed that wild-type p53 induced GDF-15 secretion, and p53-induced GDF-15 secretion was abolished by treatment with GDF-15 siRNA. These results indicate that in the normal condition, SKOV3 and PC3 cells cannot secret GDF-15, and the level of GDF-15 secretion can be regulated by p53. In addition, our results suggest that the inhibitory effects of p53 on the cell motility may be partially mediated by the secreted GDF-15. Here, we found that GDF-15 was efficiently induced by wild-type p53 but not by p53 mutants deficient in DNA binding (V143A, R175H, R248W, and R273H) (21) in two p53-null cancer cell lines. These results suggest that the transcriptional activity of p53 is required for the p53-induced GDF-15 expression. Mutations in p53 are the most common genetic abnormalities in human cancer, predominantly through missense mutations that result in accumulation of mutant p53 protein. These mutations may confer dominant-negative or gain-of-function properties to p53 (38). Missense mutations in the p53 DNA-binding domain occur in a large fraction of human tumors and have been found in over 50 different types of cancer. Large deletions, although common in other tumor suppressor genes, occur rarely in p53 (39). In the present study, we used two p53-null cancer cell lines, SKOV3 and PC3, as our experimental models and showed that overexpression of wild-type p53 induced GDF-15 expression and secretion. However, whether wild-type p53 retains the inductive effect on GDF-15 in the cells with wild-type or non-null mutations remains unknown. Future studies addressing this issue will be of interest.

In conclusion, we showed that overexpression of wild-type p53 attenuates cancer cell motility, which required p53-induced GDF-15 expression and secretion. In addition, our results indicate that GDF-15 mediates the functions of p53 by autocrine/paracrine action. These results increase our understanding of the functions of the tumor suppressor p53 and will be useful in the development of new strategies to prevent tumor metastasis.
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