Down-regulation of Phosphoglucose Isomerase/Autocrine Motility Factor Expression Sensitizes Human Fibrosarcoma Cells to Oxidative Stress Leading to Cellular Senescence

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Phosphoglucose isomerase-autocrine motility factor (PGI/AMF) is a housekeeping gene product present in all cells, is an essential enzyme of catabolic glycolysis and anabolic gluconeogenesis, and regulates tumor cell growth and metastasis. Because glycolytic enzyme up-regulation of expression contributes to glycolytic flux, leading to increased cell growth and a resistance to cellular stress of normal fibroblasts whereas down-regulation of PGI/AMF leads to mesenchymal-to-epithelial transition in tumor cells, we examined the involvement of PGI/AMF in overcoming cellular senescence in cancer cells. PGI/AMF cellular expression in HT1080 human fibrosarcoma was down-regulated by small interfering RNA methodology, which resulted in an increased sensitivity to oxidative stress and oxidative stress-induced cellular senescence. Signaling analysis revealed that the senescence pathway involving p21 cyclin-dependent kinase inhibitor was up-regulated in PGI/AMF knockdown cells and that superoxide dismutase is the upstream regulator protein of p21-mediated cellular senescence. A specific inhibitor of PGI/AMF induced cellular senescence and p21 expression in tumor cells exposed to an oxidative stress environment. Taken together, the results presented here suggest that PGI/AMF is involved in oxidative stress-induced cellular senescence and should bring novel insights into the control of cellular growth leading to a new methodology for cancer treatment.
the above implies that PGI/AMF is involved in cellular senescence; however, its signaling pathway remains elusive. Thus, we investigated the role of PGI/AMF in cellular responses to oxidative stress-induced senescence.

**EXPERIMENTAL PROCEDURES**

Reagents and Antibodies—Erythrose 4-phosphate, MTT, and anti-β-actin were obtained from Sigma. The reactive oxygen species (ROS) indicator 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) was purchased from Molecular Probes (Eugene, OR). Anti-Akt and -phospho-Akt (Ser473) were from Cell Signaling Technology (Beverly, MA). Anti-copper-zinc superoxide dismutase (CuZnSOD) and manganese superoxide dismutase (MnSOD) were from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-p21 was from Transduction Laboratories (Lexington, KY). Anti-p53 and -MDM2 were from Calbiochem (La Jolla, CA). Anti-PGI/AMF was provided by Pfizer, Inc. (New York, NY).

**Cell Lines and Culture**—Human fibrosarcoma HT1080 cells (ATCC CCL-121) were purchased from the American Type Culture Collection (Manassas, VA) and were cultured in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin. Human breast cell lines MCF10A, MCF10AT1, and DCIS.com cells were obtained from Karmanos Cancer Institute (originally called Michigan Cancer Foundation). MCF10A and MCF10AT1 cells were maintained in Dulbecco’s modified Eagle’s medium with Ham’s F-12 medium supplemented with 0.1 μg/ml cholera toxin, 0.02 μg/ml epidermal growth factor, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 100 units/ml penicillin, 100 μg/ml streptomyein, and 5% horse serum. DCIS.com cells were maintained in Dulbecco’s modified Eagle’s medium with Ham’s F-12 medium supplemented with 5% horse serum. The cultures were maintained at 37 °C in an air/5% CO₂ incubator.

**siRNA Down-regulation of PGI/AMF and SODs**—Preparation of HT1080 cells expressing a siRNA-targeting PGI/AMF (siPGI/AMF) was described previously (26). The target sequences for the SODs siRNA were: 5′-AAGGCCUGCAUGGAUCCAUG-3′ (for human CuZnSOD) and 5′-AAAUUGCGUCGUUGUACAAUC-3′ (for human MnSOD). Transfections were performed as described previously (26).

**H₂O₂ Treatment**—H₂O₂ treatment was carried out 24 h after seeding by incubating cells in the culture medium containing H₂O₂ (0–200 μM) at 37 °C for 2 h.

**Protein Extraction**—For whole cell lysates, the cells were washed twice with phosphate-buffered saline and collected by scraping. The cell pellets were lysed in cold precipitation assay buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% of Nonidet P-40, Triton X-100, sodium deoxycholate) containing 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The samples were clarified by centrifugation (15,000 rpm in 4 °C for 30 min). The cell supernatants were 100-fold concentrated with Amicon

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**FIGURE 1.** PGI/AMF knockdown cells are sensitive to oxidative stress. A, HT1080 cells were stably transfected with plasmid containing PGI/AMF-specific siRNA (siPGI/AMF) or control siRNA (mock). The cells were then analyzed by immunoblot analysis for PGI/AMF and β-actin expression. Representative results of three different experiments are shown. B, cellular senescence was assessed by the SA β-gal activity. The cells were seeded and treated with H₂O₂ for 2 h after seeding. After removing H₂O₂ by changing medium, the cells were incubated in fresh culture medium for 8 days and stained with SA β-gal. C, time course of SA β-gal activity assay. The cells were seeded and treated with 150 μM H₂O₂ for 2 h. After removing H₂O₂ by changing medium, the cells were incubated for indicated time points and stained with SA β-gal. The data are presented as the means ± S.E. for triplicate determinations. *, p < 0.05 compared with each control. D, representative SA β-gal assay of 150 μM H₂O₂-induced senescent cells.
PGI/AMF Regulates Cellular Senescence

Ulra (30,000 NMWL; Millipore, Bedford, MA). Protein concentrations of each sample were determined using Bio-Rad protein assay reagent.

Western Analysis—Western analysis was performed as described previously (26). Equal amounts of the proteins were separated on SDS-PAGE gels and transferred to a 0.2-μm polyvinylidene fluoride membrane (Osmonics Inc., Minnetonka, MN) at 15 V, 30 mA overnight at 4 °C. Then the membrane was blocked with 0.1% casein solution in 0.2× phosphate-buffered saline for 1 h at room temperature. The blocked membrane was incubated with primary antibody, and then secondary antibodies were conjugated with fluorophore (IRDye 800 antibodies (Rockland Immunochemical, Gilbertsville, PA) or Alexa-Fluor 680 antibodies (Molecular Probes, Eugene, OR)). The blots were visualized by using the LI-COR Bioscience Odyssey Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

Senescence-associated β-Galactosidase Assay—The SA β-Gal activity was monitored using a senescence detection kit (Calbiochem), and cells were observed under an Olympus fluorescence microscope.

MTT Assay—MTT assay was used to determine cell proliferation and viability. Briefly, 1 × 10^3 cells/well were plated in 96-well plates and cultured for different time periods indicated. At the end of the assay time period, 10 μl of MTT (5 mg/ml) was added to each well and then incubated at 37 °C for 4 h. After removing the medium completely, solubilizing solution (acidic isopropanol) was added at 100 μl/well. The plates were read at 570 nm on a spectrophotometric plate reader with a reference wavelength at 650 nm.

Detection of ROS Production—Cells seeded on glass culture slides (Nalge Nunc International Co., Naperville, IL) were washed with phosphate-buffered saline and loaded with 1 μM CM-H2DCFDA in phosphate-buffered saline for determining the intracellular ROS level, for 15 min at 37 °C in the dark. Dichlorofluorescein-dependent fluorescence was analyzed in an Olympus fluorescence microscope using a X400 lens.

Statistical Analysis—The data are expressed as the means ± S.D. or S.E. Comparisons between the groups was determined using unpaired t test. p < 0.05 was considered statistically significant.

RESULTS

PGI/AMF Knockdown Induces Cellular Senescence—To study the possible role of PGI/AMF in cellular senescence, we partially knocked down PGI/AMF in HT1080 fibrosarcoma cells by gene silencing using siRNA duplexes derived from the human PGI/AMF sequence (Fig. 1A; complete silencing of PGI/AMF protein expression results in loss of viability). Transfection of anti-PGI/AMF siRNA markedly reduced the protein level of PGI/AMF in HT1080 cells (>50%), whereas the control siRNA did not affect the PGI/AMF protein level of expression (Fig. 1A).

Cellular senescence is a cell-triggered program in response to a variety of stresses including oxidative stress such as exposure to H₂O₂, which induces premature cellular senescence (2–4, 28). To assess the knockdown effect of PGI/AMF on senescence, the cell transfectants were exposed to H₂O₂, and the senescence biomarker, SA β-gal activity, was measured. The SA β-gal activity was increased in a dose-dependent manner in response to treatment with H₂O₂, and HT1080 cells transfected with anti-PGI/AMF siRNA exhibited a higher rate of SA β-gal activity as compared with the control cells (Fig. 1B). siPGI/AMF cells showed about a 2-fold higher SA β-gal-positive cell formation than the control. Time course experiments revealed that SA β-gal-positive cell formation was detected in HT1080 cells within 4 days after exposure to 150 μM H₂O₂ and was highly increased in siPGI/AMF cells at all of the time points (Fig. 1C). Fig. 1D shows an example of SA β-gal assay of 150 μM H₂O₂-induced senescent cells at day 8. Furthermore, the cellular response to H₂O₂ as measured by cell proliferation revealed that such a treatment lead to a dose-dependent retardation of cell growth, and the siPGI/AMF cells showed significantly lower growth rate and greater sensitivity to H₂O₂ than the controls (Fig. 2). Similar results were obtained with another clone of PGI/AMF knockdown cells (siPGI/AMF-2; supplemental Figs. S1 and S2).

Next, we examined whether the above results were limited to HT1080 cells or are a general phenomenon. Thus, we analyzed the breast cancer progression cell model that provides a unique tool for the investigation of molecular changes associated with progression of human breast cancer as it depicts early and late stages of human breast cancer, i.e. MCF10A cell (nontumori-

![FIGURE 2. Influence of oxidative stress on cell growth.](image-url) Cellular growth under oxidative stress was determined by MTT assay. HT1080 cells were seeded at low density, treated with H₂O₂ for 2 h, and grown for 8 days. The data are presented as the means ± S.D. for triplicate determinations.

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cells (Fig. 3E) and found that the MCF10A cells were ~2.5-fold more sensitive to oxidative stress than the counterpart cells (Fig. 3B). Next, using the PGI/AMF-siRNA technique, we found that both endogenous and exogenous expressions were suppressed in DCIS.com cells (Fig. 3C) associated with a 2-fold increase in SA β-gal-positive cells formation than the control, not only under oxidative stress condition but also under normal conditions (Fig. 3D). Furthermore, we checked the effect of PGI/AMF knockdown for cellular senescence in MCF10A cells (Fig. 3, E and F). In PGI/AMF knockdown MCF10A cells, a similar pattern was observed for oxidative stress-induced cellular senescence, but the differences were not statistically significant (Fig. 3F). These results suggest that PGI/AMF involvement in the cellular senescence process is not cell type-specific, and exogenous PGI/AMF is required for regulating cellular senescence.

The effect of PGI/AMF knockdown on cell cycle proteins expression under oxidative stress. Cellular senescence is the state of stable cell cycle arrest induced by oxidative stress, DNA damage, and oncogene activation and is thought to be a tumor suppressor mechanism (2–4). To explore the molecular mechanisms underlying cellular senescence in PGI/AMF knockdown tumor cells, we studied the expression of cell cycle regulatory proteins and antioxidant proteins. As compared with the HT1080 control cells, p53 expression was significantly elevated in siPGI/AMF cells followed by induction of a target of p53, e.g., p21 (Fig. 4A). It is now well accepted that the enhanced activities of antioxidative enzymes, such as SOD, play an essential role in preventing the development of cancer (32–34). Thus, we examined their level in response to PGI/AMF down-regulation in HT1080 cells. The levels of both of CuZnSOD and MnSOD enzyme were increased ~2-fold in these cells as compared with the control cells (Fig. 4B). Consistent with the increased level of SODs, siPGI/AMF tumor cells exhibited a
marked decrease of ROS generation (Fig. 4C), a factor known to stimulate cell proliferation and induce genetic instability, are elevated in cancer cells, and are eliminated from the cells by SOD (35).

Akt protein is involved in several regulatory pathways controlling cell proliferation and survival and is thought to play an important role during cancer progression (36); thus, we have analyzed its expression. Although expression level was not changed in the siPGI/AMF cells as compared with control cells, its phosphorylation level was almost completely abolished (Fig. 4D). These results give credence to the prior results showing that overexpression of PGI/AMF in NIH-3T3 fibroblasts leads to accumulation of cellular phosphorylated Akt protein (24).

Next, we tested the effect of down-regulation of PGI/AMF expression on the expression level of MDM2 protein, which is phosphorylated by Akt, inhibits the transcriptional activity of p53, and induces p53 degradation as an E3 ubiquitin ligase (36, 37). Unexpectedly, MDM2 protein was not decreased, but rather increased in siPGI/AMF cells (Fig. 4E), suggesting that up-regulated p53 by PGI/AMF knockdown may stimulate the expression of MDM2 (p53-MDM2 feedback loop; Ref. 37).

To verify our proposed signaling pathway, PGI/AMF knockdown cells were transfected with siRNA specific to the SODs, which is the most upstream factor in our proposed pathway. Western blot analysis revealed that knockdown of SODs by siRNA down-regulated p53 and p21 protein expressions and up-regulated ROS and phosphorylated Akt levels, although SOD knockdown did not affect PGI/AMF and MDM2 expressions (Fig. 5A and B). Moreover, suppression of MnSOD, not CuZnSOD, in PGI/AMF knockdown cells showed a significantly lower rate of cellular senescence as compared with the control siPGI/AMF cells (Fig. 5C). These results suggest that SOD is upstream factor of p21, and SOD expression, especially MnSOD expression, has an important role in oxidative stress-induced cellular senescence.

We proceeded to explore the expression of p21 protein, a critical mediator of cellular senescence, and its expression level was markedly increased following treatment with H$_2$O$_2$ (Fig. 6). In addition, CuZnSOD and MnSOD protein levels were clearly induced by H$_2$O$_2$ treatment in both control and PGI/AMF knockdown cells (Fig. 6). The level of MnSOD was highly elevated by H$_2$O$_2$ treatment in siPGI/AMF cells, whereas CuZnSOD showed a slight increase in the H$_2$O$_2$-treated siPGI/AMF cells, and the same level
of CuZnSOD expression was seen in both treatment groups (Fig. 6). Because the level of p21 expression was correlated with that of MnSOD level, we suggest that PGI/AMF knockdown increases sensitivity to oxidative stress.

**Effect of PGI/AMF Inhibitor on Cellular Senescence**—Because PGI/AMF can be neutralized by specific inhibitor carbohydrate phosphates that inhibit both enzymatic and cytokine activities (10, 38), we examined whether carbohydrate phosphates could induce cellular senescence in HT1080 cells. In the presence of the carbohydrate phosphate erythrose 4-phosphate, an increase in the number of SA β-gal-positive cells was observed following exposure to the H$_2$O$_2$ oxidative stress (Fig. 7A). Moreover, MnSOD and p21 expression levels were up-regulated by erythrose 4-phosphate treatment with H$_2$O$_2$ exposure (Fig. 7B). These results also support the notion that PGI/AMF expression/activity is involved in cellular senescence response to oxidative stress, and MnSOD is a downstream factor of PGI/AMF in oxidative stress-induced cellular senescence.

**DISCUSSION**

PGI/AMF has been reported to regulate proliferation and survival of cells and prevent stress-induced apoptosis in tumor cells (24, 26, 27). Here, we report that PGI/AMF knockdown induces cellular senescence in tumor cells in response to oxidative stress by investigating SA β-gal expression, a well known marker of cellular senescence. This is also supported by the observation that PGI/AMF inhibitor induces senescence in tumor cells under oxidative stress conditions. To study this phenomenon, we have used the human fibrosarcoma cell line HT1080, which harbors wild-type p53 protein (39). Wild-type p53 is a tumor suppressor protein, is a transcription factor that activates the transcription of downstream effectors genes and affects cell cycle arrest at the G1 and G2 checkpoints in response to DNA damage. Among the p53-inducible genes, the cyclin-dependent kinase inhibitor p21 is thought to be a critical mediator of cellular senescence (4, 6, 40). The data presented here show that down-regulation of PGI/AMF induces p21 expression in tumor cells and is markedly enhanced under oxidative stress conditions, suggesting that PGI/AMF is an important regulator in the senescence program through the p21 pathway.

What signaling molecules are upstream of p21? The data indicate that SOD expression, especially that of MnSOD, is up-regulated by oxidative stress under conditions of insufficient expression of PGI/AMF, and therefore the generation of ROS is decreased because of the increase of its scavenger (35). Reduction of ROS leads to a decrease in Akt activation (41, 42), resulting in up-regulation of p21 expression (36, 43), which promotes cellular senescence. In addition, it may be suggested that p53 initiates senescent growth arrest at least in part by inducing p21, and elevated p53 may induce MDM2 expression, which in turn binds p53 and promotes p53 degradation via the ubiquitin-proteasome pathway (36, 37). Thus, no simple mechanism regulates cellular senescence, and based on our data we suggest the pathway in Fig. 8.

Many tumors exhibit strong suppressing function(s) on cellular antioxidant defense systems such as SOD, catalase, glutathione, etc., and one of the most effective intracellular enzymatic antioxidants is SOD. SOD exists in several isoforms in humans, and cytosolic CuZnSOD and mitochondrial MnSOD are two well known subtypes (44). The activity of total SOD (CuZnSOD and MnSOD) has also been found to be reduced for certain tumor cells (32). Cancers from a variety of cell types have been reported to exhibit decreased MnSOD activity (32), overexpression of MnSOD leads to tumor growth retardation (45), and elevated MnSOD activity is correlated with a loss of metastatic abilities of tumor cells (33). Although it is clear that MnSOD has tumor-suppressing activity, the mechanism of action is still unclear. At least one mechanism that MnSOD decreases tumor growth is by causing a delay at G0/G1 in the cell cycle (46). We show here that SOD expression could be induced under oxidative stress condition and was controlled by PGI/AMF. In addition the data show that ROS production was fol-
PGI/AMF Regulates Cellular Senescence

![Diagram showing oxidative stress-induced molecular signaling on cellular senescence under insufficient PGI/AMF conditions.](image)

In conclusion, the data signify that PGI/AMF plays a regulatory role in the control of cellular senescence and suggest that cellular senescence by oxidative stress is induced, in part, via the PGI/AMF pathway, and that the function of p21 cyclin-dependent kinase inhibitor during senescence is PGI/AMF-dependent. The data presented here provide a novel insight not previously described on the understanding of cellular senescence, suggest a novel therapeutic target, and underscore the role of glycolysis and gluconeogenesis enzymes in the pathobiology of the cell.

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