Transforming growth factor-β1 induces glutathione peroxidase-1 and protects from H_2O_2-induced cell death in colon cancer cells via the Smad2/ERK1/2/HIF-1α pathway

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Abstract. Recent studies have shown that transforming growth factor-β1 (TGF-β1) signaling plays important roles in the redox system in benign and malignant cells. Whether TGF-β mediates an antioxidative damage response in colorectal cancer cells is largely unknown. Herein, using the human colorectal cancer cell lines we found that TGF-β1 induced glutathione peroxidase-1 (GPx-1) expression and enzyme activity, and that the upregulation of GPx-1 by TGF-β1 could protect colorectal cell lines from H_2O_2-induced oxidation damage. Further, we used loss- and gain-function approaches to elucidate the underlying mechanism and found that TGF-β1 induced GPx-1 through activation of the TGF-β receptor type I (TGF-βRI)/Smad2/extracellular-signal-regulated kinase 1/2 (ERK1/2)/hypoxia-inducible factor-1α (HIF-1α) signaling pathway. This cascade could be blocked by the TGF-βRI inhibitor or ERK1/2 inhibitor. Taken together, our data demonstrated that TGF-β1 induced GPx-1 expression and enzymatic activity via the TGF-βRI/Smad2/ERK1/2/HIF-1α signaling pathway, suggesting a novel antioxidative protective function of TGF-β1 in colorectal cancer cells.

Introduction

Transforming growth factor-β (TGF-β) is a multifunctional cytokine and plays very important roles in development, differentiation and in maintaining homeostasis in almost all cell types and tissues (1). TGF-β functions via transmembrane serine/threonine kinase receptors, the type I and type II receptors (TGF-βRI and TGF-βRII) (2) and intracellular Smad transcriptional regulators (3). Increasing evidence has indicated that TGF-β is involved in the redox system in benign and tumor cells, in terms of that TGF-β can induce production of reactive oxygen species (ROS) which stimulates proliferation, invasion, migration and angiogenesis and evade apoptosis in cancer cells (4). TGF-β1 can stimulate a rapid increase in ROS generation to induce kidney myofibroblast activation and matrix synthesis (5). In renal tubular epithelial cells, TGF-β1 induces cellular ROS and mediates the epithelial-mesenchymal transition (EMT). All these effects can be abrogated by antioxidants (6). Low levels of ROS play a very important role in keeping normal cell proliferation by regulating cellular signaling. A great body of evidence indicates that production of ROS is increased in cancer cells and elevated ROS may facilitate tumor cell growth (7). However, a higher level of ROS may act reversely to induce an apoptotic response of tumor cells. Moderate ROS levels provide tumor cells with the advantage to maintain survival. Glutathione peroxidases (GPxs) are of the most important antioxidative enzyme families which can modulate cellular ROS levels to keep the redox balance in tumor cells.

GPx-1 is the first identified selenoprotein and is expressed in most cells (8-10). GPx-1 is classified as an antioxidant enzyme and exerts its role to prevent the initiation of cancer by ROS-mediated DNA damage. Previous studies have suggested that GPx-1 is altered in several types of cancer cells (11,12) and overexpression of GPx-1 shows antitumorigenic effect by eliminating oxidants (13). On the other hand, GPx-1 also increases tumorigenesis in transformed cells by limiting apoptotic mechanisms (14,15). It has been reported that TGF-β can suppress catalase and GPx-1 mRNA expression and reduce their antioxidative activities in chronic pancreatitis and rat hepatocytes (16,17). However, whether there is a regulatory interaction between TGF-β and GPx-1 in colorectal cancer cells remains unclear.

Another factor that participates in the redox balancing regulation is the hypoxia-inducible factor-1α (HIF-1α). HIF-1α is an important transcriptional modulator in oxygen-regulated gene expression. HIF-1α is sensitive to hypoxia, but it can

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Abbreviations: TGF-β1, transforming growth factor-β1; GPx-1, glutathione peroxidase-1; HIF-1α, hypoxia-inducible factor-1α; ERK1/2, extracellular-signal-regulated kinase 1/2

Key words: TGF-β1, Smad2, glutathione peroxidase-1, HIF-1α, ERK1/2, colon cancer

also be regulated under the condition of normoxia. TGF-β1 can induce HIF-1α stabilization through selective inhibition of prolyl hydroxylase 2 (PHD2) expression (18). Whether HIF-1α is involved in TGF-β1-induced alterations of GPx-1 has not been reported.

In this study, we investigated the antioxidative protective function of TGF-β1 in colorectal cancer cells and the underlying mechanism. We found that TGF-β1 induced GPx-1 expression at the protein levels and induced GPx-1 activity in DLD-1 colorectal cancer cells, subsequently, to protect cancer cells from exogenous H_2O_2-induced death, although TGF-β1 did not affect GPx-1 mRNA levels. In addition, we found that TGF-β1 could induce HIF-1α expression via activating the TGF-βRI/Smad2/extracellular-signal-regulated kinase 1/2 (ERK1/2) signaling pathway, leading to overexpression of GPx-1 protein. Taken together, TGF-β1 induced GPx-1 expression and its enzyme activity via activating TGF-βRI/Smad2/ERK1/2/HIF-1α signal cascades, resulting in the protection of DLD-1 colorectal cancer cells from oxidative damage.

Materials and methods

Cell culture and reagents. Human colorectal adenocarcinoma DLD-1 cells and HCT116 cells were purchased from China Center for type culture collection (CCTCC, Wuhan University, Wuhan) and were cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (Hyclone, Beijing, China), 1% antibiotic-antimyocytic (Invitrogen, Grand Island, NY, USA) and maintained at 37˚C in a humidified incubator with 5% CO_2. TGF-β1 (Invitrogen) and cobaltous chloride ($CoCl_2$) (Sigma, Switzerland) were used for cell culture.

Transfection. Transient transfection of DLD-1 cells with HA-HIF-1α P402A/P564A-pcDNA3 (HA-HIF-1α-Mut) (Addgene, USA), pcDNA3.1 vector control, HIF-1α-siRNA and GFP-siRNA were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Thirty-six hours after transfection, cells were subjected to different assays. siRNA duplex oligonucleotides were synthesized by RiboBio Co., Ltd. (Guangzhou, China). The sequence for the HIF-1α-siRNA was 5'-CUGAUGACCAGCAACUUGAdTdT-3' (19). A scrambled-siRNA GFP-siRNA was used as control. The cells were treated with or without TGF-β1 (5 ng/ml) for 6 h after 36 h transfection of siRNA. Cell lysates were collected for western blot analysis.

Western blot analysis. At the end of incubation, cells were washed with cold PBS and lysed in 70 μl of lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, sodium pyrophosphate, EDTA, β-glycerophosphate, Na_3VO_4, leupeptin, and protease inhibitors) (Beyotime Biotechnology, China) for 10 min at 4˚C. Cell lysates were centrifuged at 12,000 rpm at 4˚C for 10 min to pellet cell debris. The concentration of cellular protein was determined using the BCA Protein Assay kit (Beyotime Biotechnology). Total protein of 60 μg was mixed with a 5X loading buffer (60 mM Tris-HCl , pH 6.8, 25% glycerol, 2% SDS, 0.1% bromophenol, and 0.2% 2-mercaptoethanol) and heated at 100˚C for 5 min and were separated on 12% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred onto a Millipore Immobilon-P transfer membrane (Millipore Corporation, USA) using a Semi-Dry system (Bio-Rad Laboratories, USA) with Tris buffer (0.025 M Tris-HCl, 0.192 M glycine, and 20% MeOH). The membrane was blocked for 1 h at room temperature with 5% nonfat milk in TBS-Tween-20. Membranes were incubated at 4˚C overnight with the following antibodies: mouse monoclonal antibody to β-actin (Santa Cruz Biotechnology, Inc., 1:2,000), rabbit polyclonal GPx-1 antibody (Abcam, 1:1,000), mouse monoclonal Smad2 (Santa Cruz Biotechnology, Inc., 1:500), rabbit polyclonal phospho-Smad2 (Ser465/467) antibody (1:1,000), rabbit polyclonal p38 MAPK antibody (1:800), rabbit polyclonal phospho-p38 (Thr180/Tyr182) MAPK antibody (all from Cell Signaling Technology, Inc., 1:1,000), mouse monoclonal JNK1 (Santa Cruz Biotechnology, Inc., 1:500), mouse monoclonal p-JNK (Santa Cruz Biotechnology, Inc., 1:500), rabbit polyclonal p44/42 MAPK (ERK1/2) antibody (Cell Signaling Technology, Inc., 1:500), rabbit polyclonal phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) antibody (Cell Signaling Technology, Inc., 1:500) or rabbit polyclonal HIF-1α antibody (Abcam, 1:800), respectively. After washing in TBS-Tween-20, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG for 1 h at room temperature. After washing, the membranes were incubated with BeyoECL Plus reagents (Beyotime Biotechnology). Images were captured using a motored molecular imaging system (Molecular Imaging Vilber Fusion X7, France).

Measurement of GPx activity. DLD-1 cells were pretreated with the TGF-β1RI inhibitor LY364947 (Tocris Bioscience, USA) for 1 h, then cells were incubated with or without TGF-β1 (5 ng/ml) for 6 h. Cell lysates were assayed with the Cellular Glutathione Peroxidase Assay kit (Beyotime Biotechnology) at 25˚C according to the manufacturer's instructions.

Real-time reverse transcription-PCR (RT-PCR) analysis. Real-time reverse PCR was carried out in a 25 μl total reaction mixture with 2.5 μl of extracted RNA sample (0.5 ng), 12.5 μl of One-Step reverse transcriptase (RT) qPCR master SYBR-Green mix (Toyobo Co., Japan), 10 pM forward primer (1 μl), 10 pM reverse primer (1 μl), and 8 μl of RNase-free distilled water. Amplification and detection were performed by the real-time PCR Detection System (Rotor-Gene 3000, Corbett Research, Australia) under the following conditions: an initial reverse transcription at 70˚C for 15 min, followed by PCR activation at 95˚C for 1 min and 40 cycles of amplification (15 sec at 95˚C and 45 sec at 72˚C). During amplification, a detector monitored real-time PCR amplification by quantitative analysis of the fluorescence emissions. Sample values obtained with specific primer sets were normalized to β-actin primer set values. For qRT-PCR, the following primer were used: β-actin, forward, 5'-GTCCACCGCAAATGCTTCTA-3' and reverse, 5'-TGCTGTCACCTTCACCGTTC-3'; GPx-1, forward, 5'-CC AAGCTCATCACCTGGTCT-3' and reverse primer, 5'-TCGAT GTCAATGGTCTGGAA-3' as recently described by us (20).

Cytotoxicity of H_2O_2. Following incubation with or without TGF-β1 (5 ng/ml) and LY364947 for 6 h, the cells were exposed to different concentrations of H_2O_2 for 24 h. Then the cytotoxic effects of H_2O_2 were analyzed by the MTS assay

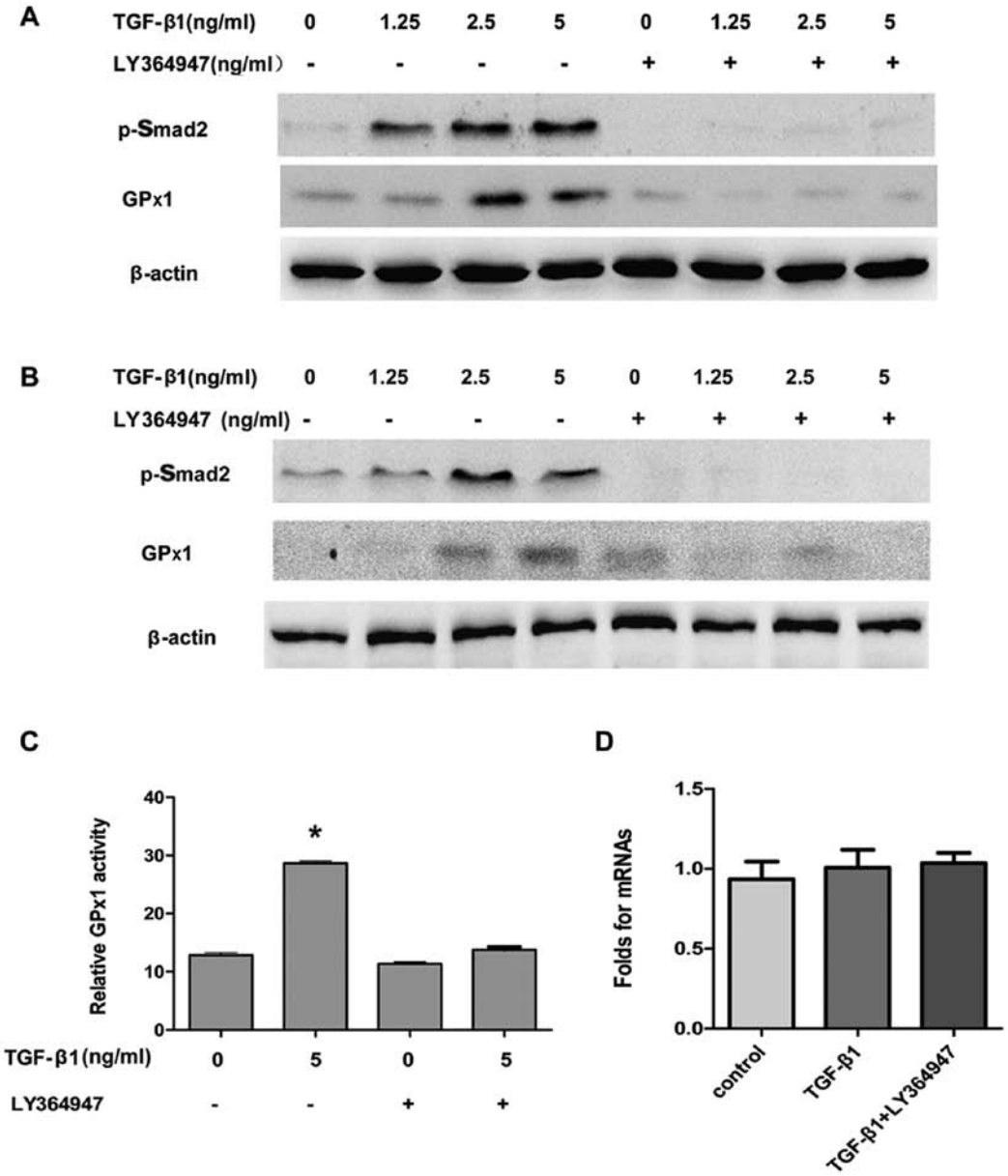

Figure 1. GPX-1 is induced by TGF-β1 via the TGF-β type I receptor in colorectal cancer cells. DLD-1 cells and HCT116 cells were pre-incubated in the absence or presence of the TGF-β1 receptor I inhibitor LY364947 for 1 h, then treated with different concentrations of TGF-β1 for 6 h. Cell lysates were collected for analysis. Dose-dependent response of GPx-1 protein expression was performed in (A) DLD-1 and (B) HCT116 cells, assayed by western blotting. (C) Effects of TGF-β1 on GPx-1 activity. (D) Colorectal cancer cells were pre-incubated in the absence or presence of LY364947 for 1 h. Cells then were treated with or without 5 ng/ml TGF-β1 for 6 h. Total-RNA of conditioned cells were collected and analyzed by RT-PCR. (C and D) Data are expressed as mean ± SD of at least three independent experiments (n=3). (C) *P<0.05, as compared with the controls. The image represents one of three similar experiments (n=3).

according to the manufacturer's protocol (Promega, Madison, WI).

Statistical analysis. Results were presented as mean ± SD and analyzed with GraphPad Prism 5. ANOVA was used for the comparisons among groups. P<0.05 was considered statistically significant. All experiments were repeated at least 3 times.

Results

TGF-β1 induces GPx-1 expression via a TGF-β type I receptor in colorectal cancer cells. To investigate the effects of TGF-β1 on GPx-1 expression, two colorectal cancer cell lines, DLD-1 and HCT116 were pretreated with a selective TGF-β1 receptor I (TGF-βRI) inhibitor LY364947 for 1 h, then incubated with various concentrations of TGF-β1 (0, 1.25, 2.5, 5 ng/ml) for 6 h. Cell lysates were collected and analyzed by gelatinzymography, cellular glutathione peroxidase assay and real-time PCR, respectively. TGF-β1 induced GPx-1 expression in a concentration dependent manner in DLD-1 cells (Fig. 1A). There was an apparent upregulation when treated with 5 ng/ml TGF-β1 within 6 h. Similar results were seen in the HCT116 colon cancer cells (Fig. 1B). Subsequent experiments were performed on the DLD-1 cells.

To investigate whether there was a change of TGF-β1-induced GPx-1 activity, we performed a cellular glutathione

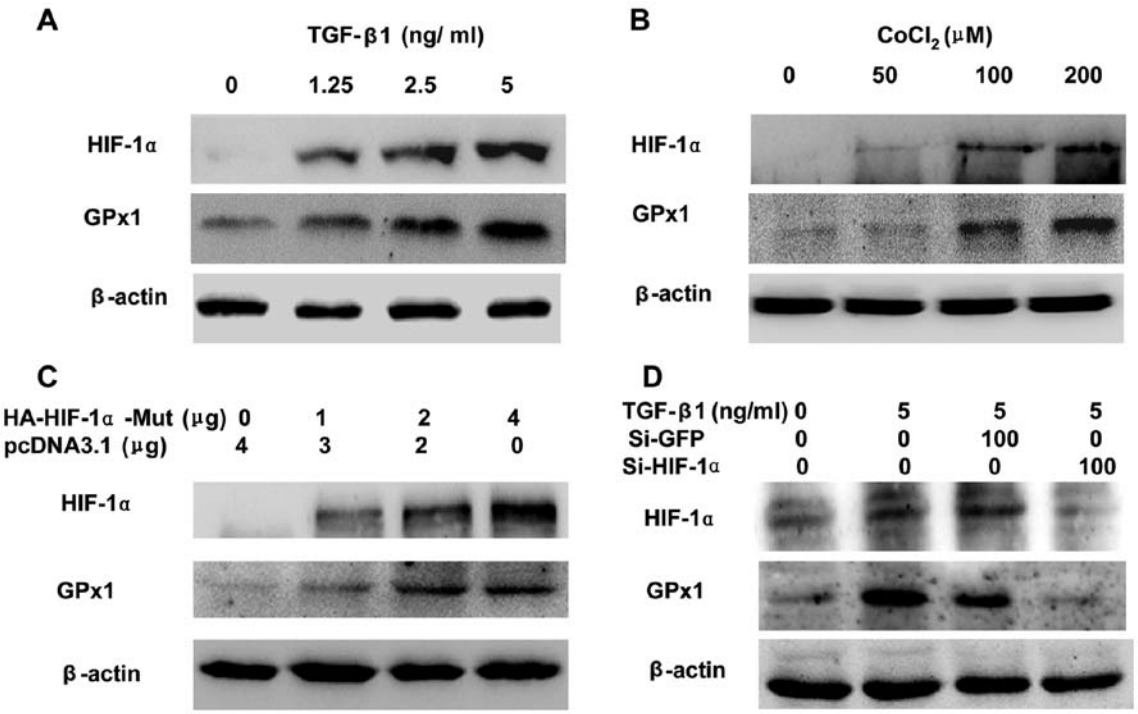

Figure 2. Involvement of HIF-1α-in TGF-β1-induced GPx-1 expression. DLD-1 cells were cultured in the presence or absence of (A) TGF-β1 or (B) $CoCl_2$ for 6 h. Cell extracts were analyzed by western blotting. (C) DLD-1 cells were transiently transfected with incremental concentrations of HA-HIF-1α P402A/P564A-pcDNA3 (HA-HIF-1α-Mut) or pcDNA3.0 empty vector (control). Cell lysates were analyzed by western blotting after transfection for 48 h. (D) DLD-1 cells were transiently transfected with incremental concentrations of HIF-1α-siRNA or control GFP-siRNA. Following 42 h transfection 5 ng/ml TGF-β1 was added into cells and incubated for 6 h. Then cell lysates were collected for western blotting. The image represents one of three similar experiments (n=3).

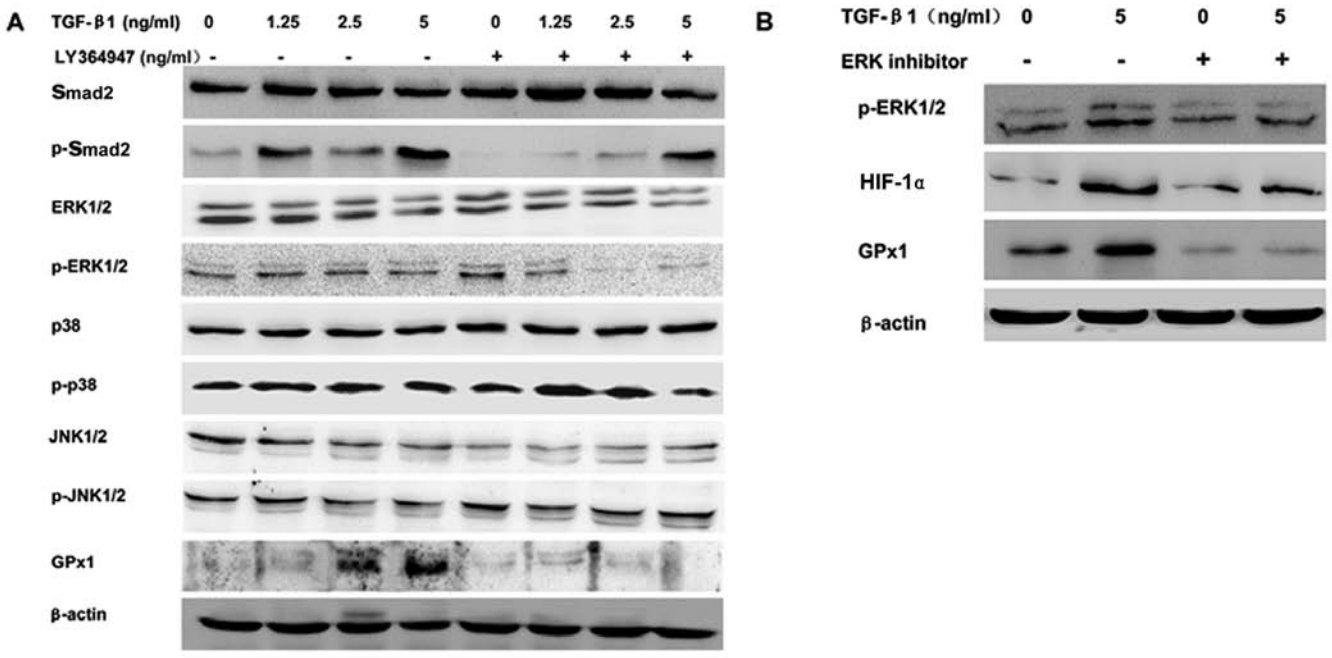

Figure 3. TGF-β1-HIF-1α-induce GPx-1 expression through ERK1/2. (A) DLD-1 cells were pretreated in the absence or presence of LY364947 for 1 h. Cells then were treated with different concentrations of TGF-β1 for 6 h. Cell lyates were collected for western blotting. (B) Cells were pre-treated with or without ERK1/2 inhibitor for 1 h, followed by 5 ng/ml TGF-β1 and incubation for 6 h. Cell extracts were analyzed by western blotting.

peroxidase assay. The data showed that TGF-β1 increased GPx-1 activity when compared with controls (Fig. 1C). To further examine whether the increase of GPx-1 expression by TGF-β1 resulted from the induction of GPx-1 mRNA expression, RT-PCR analysis was performed. The data showed that TGF-β1 does not induce GPx-1 mRNA expression in DLD-1 cells (Fig. 1D). The expression of the housekeeping gene β-actin (as an internal control) mRNA was not altered. These

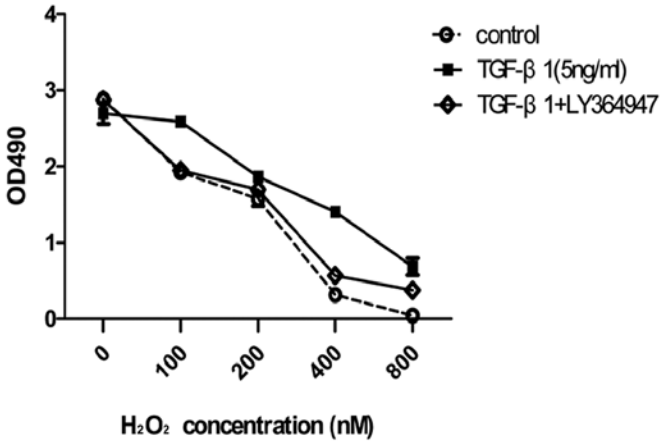

Figure 4. TGF-β1 protects DLD-1 cells from H_2O_2-induced cell death through upregulation of GPx-1. DLD-1 cells were pre-incubated in the absence or presence of LY364947 for 1 h. Cells were then treated with or without 5 ng/ml TGF-β1 for 6 h and followed by incubation with H_2O_2 for 24 h. MTS proliferation assay was performed afterwards.

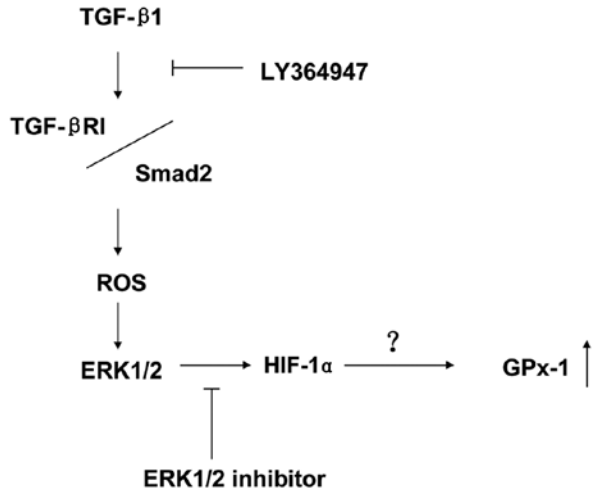

Figure 5. A proposed signaling model for TGF-β1-induced GPx-1 expression. Based on our current findings we proposed that TGF-β1-induced GPx-1 expression and activated GPx-1 enzyme activity through a TGF-β1/TGF-βRI/Smad2/ERK1/2/HIF-1α signaling cascade.

data indicated that the upregulation of GPx-1 induced by TGF-β1 occured at the post-transcriptional level. Moreover, we examined whether TGF-βRI was involved in TGF-β-induced GPx-1 expression. We treated DLD-1 cells with a selective TGF-βRI antagonist LY364947. LY364947 is a diheteroaryl-substituted pyrazole compound used as a selective, ATP-competitive inhibitor of TGF-βRI kinase (21,22). We found that blockade of TGF-βRI by LY364947 attenuated TGF-β1-induced GPx-1 expression at the protein level but not at the mRNA level (Fig. 1A and D). These results suggest that TGF-β1-induced GPx-1 protein expression occurs through TGF-βRI in colorectal cancer cells.

Role of HIF-1α in TGF-β1-induced GPx-1 expression. We examined whether HIF-1α was involved in the induction of GPx-1 by TGF-β1 in DLD-1 cells. First, we incubated DLD-1 cells with different concentrations of TGF-β1 and cobalt chloride ($CoCl_2$), respectively, then determined the expression of HIF-1α and GPx-1 by immunoblotting. $CoCl_2$ is a hypoxia-mimetic agent and can induce hypoxia and stabilize HIF-1α protein (23,24). As presented in Fig. 2A and B, HIF-1α expression was increased following 6 h stimulation with TGF-β1 or $CoCl_2$ in a concentration-dependent manner when compared with untreated cells, along with an induction of GPx-1. The increased GPx-1 expression when HIF-1α was induced by both TGF-β1 and $CoCl_2$, suggested that HIF-1α might be an intermediary agent between TGF-β1 and GPx-1.

To determine whether the effects of TGF-β1 on the change of GPx-1 expression were mediated by HIF-1α, we overexpressed HIF-1α by transient transfection of a mutated plasmid HA-HIF-1α-Mut into DLD-1 cells. We used a HIF-1a mutant plasmid instead of a wild-type plasmid because the wild-type HIF-1a is easy to be degraded within about 5 min by hydroxylation (25,26). The expression of GPx-1 protein was induced by HIF-1α overexpression (Fig. 2C). We then knocked down HIF-1α expression in DLD-1 cells by a si-RNA-HIF-1α. As shown in Fig. 2D, reduced expression of HIF-1α attenuated the expression of GPx-1 induced by TGF-β1. These results confirmed that the induction of TGF-β1 on GPx-1 was mediated by HIF-1α.

TGF-β1-HIF-1α-mediated induction of GPx-1 expression occurs through ERK1/2. Accumulating evidence suggests a regulatory interaction between the mitogen-activated protein kinases (MAPKs) and TGF-β1, and this interaction plays critical roles in the crosstalk between Smad and the MAPK signaling pathway (27-29). There are three members in the MAPK family, including ERK1/2, JNK1/2 and p38, which are involved in cell proliferation, differentiation and apoptosis induced by TGF-β1. It has been reported that TGF-β1 can induce HIF-1α-mediated VEGF secretion, and this process may be involved in TGF-β1-stimulated-ERK1/2 activation (30). Evidence demonstrates that TGF-β1-induced myofibroblast activation works through a TGF-βRI/Smad/ROS/ERK1/2 signaling cascade (5). Therefore, we determined whether the MAPK signaling pathway was involved in TGF-β1- HIF-1α-mediated induction of GPx-1 expression. First, we stimulated DLD-1 cells with TGF-β1 at different concentrations from 0 to 5 ng/ml for 6 h. Cell lysates were collected for western blot analysis. We found that TGF-β1 activated p-Smad2 and ERK1/2 MAPK, with the exception of the activation of p38 MAPK and JNK1/2 (Fig. 3A). To further investigate the role of ERK1/2 in TGF-β1-induced HIF-1α-GPx-1 expression, we treated DLD-1 cells with an ERK1/2 inhibitor and found that ERK1/2 inhibitor suppressed TGF-β1-induced phosphorylation of ERK1/2 and the expression of HIF-1α and attenuated GPx-1 protein expression (Fig. 3B). Collectively, these results suggest that TGF-β1-induced expression of GPx-1 is mediated by HIF-1α, possibly through the TGF-βRI/Smad2/ERK1/2/HIF-1α/GPx-1 signaling cascade.

TGF-β1 protects DLD-1 cells from H_2O_2-induced cell death through upregulation of GPx-1. To determine the effect of TGF-β-induced expression on GPx-1, we treated the colon cancer cells with H_2O_2, followed by analysis of cell proliferation using an MTS assay kit according to the manufacturer's

protocol (Promega, Madison, WI). As shown in Fig. 4, TGF-β1-treated cells were found to be less sensitive to exogenous H_2O_2 than controls. When the TGF-βRI inhibitor LY364947 was added, the protective function of TGF-β1-induced GPx-1 was attenuated. This finding indicated that TGF-β1-induced GPx-1 expression could protect DLD-1 cells from the cytotoxicity of exogenous H_2O_2.

Discussion

In this study, we discovered a novel anti-oxidative protective function of TGF-β1 in colorectal cancer cells, which occured through induction of GPx-1 expression and its enzyme activity via HIF-1α, leading to a protection of colorectal cancer cells from death induced by exogenous H_2O_2.

It has been well known that TGF-β is involved in multiple biological procedures including cellular replication, differentiation, bone formation, angiogenesis, cell cycle progression and, carcinogenesis (31,32). Accumulated experimental evidence over the past decade has demonstrated that TGF-β can play dual roles in cancer depending on the cell types and stages of progression (33,34). Recently studies have revealed important roles of TGF-β in the cellular redox system. For instance, TGF-β can increase ROS production in numerous types of cells through different ways (35). The ROS levels are modulated by a set of antioxidative enzymes, particularly GPx-1. GPx-1 is highly effective in preventing ROS-mediated initiation of cancer (36). Herein we observed that GPx-1 could be modulated by TGF-β1 and this modulation occured at the post-transcriptional level, because GPx-1 mRNA levels were not affected by TGF-β1. Additionally, TGF-β1-induced GPx-1 expression was mediated by HIF-1α, evidenced by using gain- and loss-expression of a HIF-1α *in vitro* model, which has not been previously reported.

Previous studies have demonstrated the critical regulation of TGF-β1 in canonical Smad and non-Smad pathways, including activation of the extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein (MAP) kinase pathways. The tyrosine kinase Src and phosphatidylinositol 3'-kinase (PI3K), and that phosphorylation of ERK1/2 can be inhibited by blocking TGF-βRI, Smad3, or the Nox oxidases (5,37). It is well established that ROS can activate MAPKs to exert functions in proliferation, differentiation and apoptosis (38,39), and ROS-dependent activation of ERK1/2 and JNK1/2 by TGF-β1 can induce MMP-9 expression and enzymatic activity, resulting in the promotion of cell migration in RBA-1 cells (40). Our results support the involvement of an additional signaling pathway, TGF-β1/TGF-βRI/Smad2/ERK1/2/HIF-1α/GPx-1, in colorectal cancer (Fig. 5). Actually, TGF-β1/ERK1/2/HIF-1α-induced GPx-1 expression could be blocked by an ERK inhibitor (Fig. 3B), and inhibition of TGF-βRI attenuated exogenous H_2O_2-induce cell death (Fig. 4).

Taken together, we report a novel anti-oxidative protective function of TGF-β1 on colorectal cancer cells and offer support for the involvement of the TGF-β/TGF-βRI/Smad2/ERK1/2/HIF-1α/GPx-1 signaling pathway in the process. How HIF-1α mediates the regulatory interaction between TGF-β and GPx-1 is not clear, and therefore, further investigation is needed.

Acknowledgements

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