Inhibition of Heme Oxygenase-1 Increases Responsiveness of Pancreatic Cancer Cells to Anticancer Treatment

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Abstract  Heme oxygenase-1 (HO-1) is believed to represent a key enzyme for the protection of cells against "stress." Its overexpression in different types of human cancers supports the notion that HO-1 provides a growth advantage and contributes to cellular resistance against chemotherapy and radiotherapy. Given the poor survival rates of patients with pancreatic cancer due to its aggressive growth behavior and its exceptional resistance to all known forms of anticancer treatment, we have investigated the expression of HO-1 in human pancreatic cancer cells growth behavior and prognosis. Expression of HO-1 was analyzed in human pancreatic cancer samples in comparison with normal pancreas by quantitative PCR, Western blot, and confocal microscopy. The influence of radiotherapy and chemotherapy on HO-1 expression in pancreatic cancer cell lines was evaluated. Furthermore, HO-1 expression was specifically suppressed by small interfering RNA transfection and subsequently the alterations of growth behavior and resistance to anticancer treatment were tested. Human pancreatic cancer showed a 6-fold and 3.5-fold HO-1 up-regulation in comparison to normal pancreas based on mRNA and protein level, respectively (P < 0.05). Cancer tissues revealed marked HO-1 immunoreactivity in tumor cells and in tumor associated immunocytes. Treatment of the pancreatic cancer cell lines with gemcitabine or radiation strongly induced HO-1 expression. Targeted knockdown of HO-1 expression led to pronounced growth inhibition of the pancreatic cancer cells and made tumor cells significantly more sensitive to radiotherapy and chemotherapy. Therefore, specific inhibition of HO-1 expression may be a new option in pancreatic cancer therapy and may be used as sensitizer to chemotherapy and radiotherapy.

Pancreatic cancer has a very poor prognosis and at present surgical resection is still the only chance for cure (1, 2). Unfortunately, most patients are diagnosed at advanced tumor stages with metastasis already in distant organs; therefore, these patients are no longer candidates for surgical treatment. Furthermore, even after a so-called “curative resection,” the 5-year survival rate is 5% to 20%. Current chemotherapy and radiotherapy regimens are not very effective (3). Therefore, additional treatment options for advanced disease adjuvant to surgical therapy are urgently needed.

The heme oxygenase (HO) system catalyzes the degradation of heme to produce equimolar quantities of biliverdin, CO, and free iron (4). Subsequently, biliverdin is converted to bilirubin by cytosolic biliverdin reductase, and free iron is promptly sequestered into ferritin (5, 6). To date, three HO isoforms (HO-1, HO-2, and HO-3) that catalyze this reaction have been identified (7–9). HO-1 is a 32-kDa inducible heat shock protein, which is found at low levels in most mammalian tissues but is highly induced by a variety of stress stimuli, including heat shock (10), UV irradiation (11), hydrogen peroxide (12, 13), heavy metals (14, 15), hypoxia (16), and cytokines (17, 18). Recent findings indicate that HO-1 and its products possess anti-inflammatory and antiapoptotic functions (19–22). It represents a key biological molecule in the adaptive response to cellular stress. Moreover, new studies suggest that HO-1 exerts also a role in controlling growth and cell proliferation in a cell-specific manner. Elevated HO-1 expression and activity was found in various tumors such as human renal cell carcinoma (23), prostate tumors (24), and lymphosarcomas (25). In human gliomas and melanomas, HO-1 is linked to angiogenesis (26–28), and in an experimental mouse model, HO-1 accelerates pancreatic cancer growth by promoting tumor angiogenesis (28).

These findings suggest that HO-1, with its proangiogenic and growth-regulative properties, may also play a crucial role in the development and progression of pancreatic cancer. Furthermore, its anti-inflammatory and antiapoptotic activity implies that HO-1 may enhance radioresistance and chemoresistance in pancreatic cancer cells. We, therefore, hypothesized that the inhibition of HO-1 expression and activity may sensitize pancreatic cancer cells to anticancer treatment modalities.
Materials and Methods

Patients and tissue collection. Twenty-seven ductal pancreatic cancer samples were obtained from 14 male and 13 female patients (median age, 66 years; range, 36-81 years) who underwent pancreatic resections because of ductal pancreatic cancer at the University Hospital of Berne (Switzerland) and Heidelberg (Germany). According to the tumor-node-metastasis and histopathologic grading system of the International Union Against Cancer, there were four stage I, four stage II, 14 stage III, and five stage IV tumors.

Normal pancreatic tissue samples were obtained from 20 individuals (7 women and 13 men) who were free of pancreatic disease through an organ donor program. The median age of the organ donors was 46.2 years (range, 20-74 years). All normal tissue samples were obtained from the head of the organ donor's pancreas to ensure comparability with the tumor samples. In all experiments, tissue sections of normal and cancerous pancreas samples were processed simultaneously to ensure comparability of the results. Freshly removed tissue samples were immediately fixed in formaldehyde solution for 12 to 24 hours and embedded in paraffin for immunohistochemistry and confocal microscopy. Simultaneously, tissues for RNA extraction were put in RNA Later (Ambion, Huntingdon, United Kingdom) whereas tissue for protein extraction were snap-frozen in liquid nitrogen in the operating room upon surgical removal and maintained at −80°C until use. The ethical committee of the University of Bern and Heidelberg approved the study and informed consent was obtained from each patient.

Cell culture. The established human pancreatic cancer cell lines Panc-1, MiaPaCa-2, SU8686, and Colo 357 were used in these experiments. All cells were obtained from American Type Cell Collection (Manassas, VA).

The Panc-1 and Mia PaCa-2 cells were cultured in Dulbecco's modified Eagle high-glucose medium with Na pyruvate (Cell Concepts, Umkirch, Germany) supplemented with 10% fetal bovine serum (PAN Biotech GmbH, Aidenbach, Germany), 4 mmol/L Glutamine-1 (Life Technologies, Paisley, United Kingdom), and a penicillin-streptomycin solution (100 units/mL and 100 µg/mL, respectively; Life Technologies). The cells were maintained at 37°C in a humidified 5% CO₂ atmosphere for at least 72 hours before each experiment and harvested after treatment with trypsin-EDTA (Life Technologies).

The SU8686 and Colo 357 cells were cultured in RPMI 1640 with 1-glutamine (Life Technologies) supplemented with 10% fetal bovine serum (PAN Biotech) and a penicillin-streptomycin solution (100 units/mL and 100 µg/mL, respectively; Life Technologies). The cells were maintained at 37°C in a humidified 5% CO₂ atmosphere for at least 72 hours before each experiment and harvested after treatment with trypsin-EDTA (X1; Life Technologies).

Heme oxygenase-1 small interfering RNA transfection. To study the effects of HO-1 inhibition on cell viability, growth rate, induction of apoptosis, and sensitization to anticancer treatment, HO-1 expression was specifically suppressed by introduction of 21-nucleotide duplex small interfering RNA (siRNA), which targets nucleotides 612 to 630 of the HO-1 mRNA coding sequence (29). The sequences of the ribonucleosides were 5'-rGACUGCCGUCCGUCUCGCACUdTdT-3' and 5'-GCUUGAGCCGACGACGCUdGdTd-3' (Ambion, Inc., Austin, TX; ref. 30). The cells (PANC-1, MIA PaCa-2, SU8686, and Colo 357) were each plated on 6-well plates (Nunc, Roskilde, Denmark) in a humidified 5% CO₂ atmosphere for 3 days. Some cells were grown for 48 hours after transfection without any treatment to determine the effect of HO-1 suppression on cell viability and to compare these results with the cell proliferation assay.

For analysis, cells were trypsinized, harvested, and added to the corresponding supernatant in fluorescence-activated cell sorting (FACS) tubes. Then the pooled cells were pelleted, washed with cold PBS (Cell Concepts), and resuspended while vortexing in 0.4 mL of cold PBS containing 2% fetal bovine serum and 2.5 µg/mL propidium iodide (Sigma-Aldrich GmbH, Steinheim, Germany). After incubating the cells at 4°C for 10 minutes, flow cytometry with the BD LSR Immunocytometry System (BD Biosciences, San Jose, CA) was used to analyze the propidium iodide staining and to determine the percentage of viable and necrotic cells. The results are presented as a percentage of viable cells in each group.

Western blot analysis. Total proteins were extracted using Laemmli buffer from treated cultured pancreatic cancer cells and control native cells. From pancreatic tissues (normal pancreas and pancreatic cancer) total protein was obtained by lysing the appropriate tissue sample (~ 500 µg) in a buffer containing 20 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 1% NP40, and protease inhibitor cocktail for mammalian tissue 50 µL per sample (Sigma, St. Louis, MO).

Total proteins were electrophoresed in NuPage MOPS SDS Running Buffer, NuPage 10% Bis-Tris Gel (Invitrogen) at 200 V. Using the semidytransfer at 0.04 A constant for 1 hour, the proteins were blotted on to Immobilon polyvinylidene difluoride 0.45-µm pore membrane (Invitrogen). After blocking with 8% milk, the membrane was incubated in a milk solution containing anti-HO-1 mouse monoclonal antibody (BD Biosciences Transduction Laboratories, Lexington, KY). The bound antigen-antibody complex was detected by secondary peroxidase-conjugated affinity purified goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and the chemiluminescence kit (Perkin-Elmer Life Sciences, Inc., Boston, MA). The same membranes were used for blotting with anti-β-tubulin or anti-GABDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibody as an internal loading control.

The Western blot films were scanned with GS-800 Calibrated Densitometer (Bio-Rad Laboratories, Inc., Hercules, CA) and further quantified using the Quantity One Quantification Software according to the manufacturer's guidelines (Bio-Rad Laboratories).
**Immunohistochemistry.** Frozen pancreatic tissue sections (5-7 μm) were air-dried and fixed in a mixture of ice-cold acetone and 10% formaldehyde for 5 minutes at −20°C. In paraffin sections, antigen retrieval was completed by microwave cooking at 800 W for 5 minutes (10 minutes cooking at room temperature) and cooking at 400 W 2 × 5 minutes in 10 mmol/L sodium citrate buffer followed by incubation with 0.05% saponin in PBS for 20 minutes at room temperature.

Further procedures for immunohistochemical detection of HO-1 were identical for frozen and paraffin sections. Sections were incubated with a rabbit polyclonal antibody to human HO-1 (Stressgen Biotechnologies Co., Victoria, British Columbia, Canada), diluted 1:250. A mouse monoclonal anti-rabbit immunoglobulin G antibodies, diluted 1:100 (DAKO A/S, Glostrup, Denmark), was used as secondary antibody. For negative controls, incubation with full rabbit serum in dilution corresponding to the concentration of the primary antibody was used.

**Fluorescent microscopy.** The cells were plated on Teflon-coated slides and fixed by ice-cold methanol for 5 minutes and acetone for 2 minutes, before 0.05% saponin was applied for 20 minutes at room temperature. After rinsing in PBS, nonspecific binding was blocked by preincubating sections with 10% normal goat serum in TBS containing 10% γ-avenin and 0.1% BSA-c for 1 hour at room temperature. Sections were incubated with a mouse monoclonal antibody to human HO-1 (BD Biosciences Transduction Laboratories), diluted 1:25 or 1:50 in 10% normal goat serum TBS containing 0.05% Tween 20 in 4°C ON.

After rinsing thrice in TBS Tween 0.05% with 1.0% bovine serum albumin, sections were incubated for 1 hour at room temperature without light exposure with goat anti-mouse Cy3-labeled antibodies, diluted 1:400 (Jackson ImmunoResearch Laboratories). Then the slides were further incubated with Toto-3 iodide 1:1.000 (Molecular Probes, Eugene, OR). For negative controls, sections were processed as described earlier, except that the incubation with mouse IgG (DAKO) in dilution 1:10 or 1:20 was employed instead of the primary antibody.

**Heme oxygenase-1 activity.** Benzene was purchased from AppliChem GmbH (Darmstadt, Germany), and barium chloride-2-hydrate from Sigma-Aldrich (Seelze, Germany). Then cells were lyzed by adding 100 μL of 10-fold Triton 100-X to each well for 1 hour and 1.0 mL of each culture supernatant was collected into corresponding tubes, and 500 mg of BaCl2 were added to each sample. After vortexing (10-15 seconds), 1.5 mL benzene was added; tubes were vortexed vigorously (50-60 seconds) leading to the formation of a relatively stable milky-white emulsion. After centrifugation (13,000 g, 30 minutes, without cooling), the upper benzene layer was collected and the absorbance at 450 nm with reference wavelength at 570 nm was measured using a Shimadzu UV-160A spectrophotometer. In a separate tube, 1.0 mL of fresh culture medium was processed in the same way and the benzene layer was collected and used as a blank. The quantity of bilirubin produced was calculated using a molar extinction coefficient of \( E^{450} = 27.3 \text{ (mmol/L)}^{-1}\text{cm}^{-1}\).

**Statistical analysis.** The data are presented as mean ± SE or median and range. P < 0.05 was considered statistically significant. For comparison between groups, the Mann-Whitney test was employed.

## Results

**Heme oxygenase-1 is overexpressed in human pancreatic cancer.** To determine whether HO-1 plays a role in human pancreatic cancer, mRNA and protein levels in human pancreatic cancer samples (n = 27) were compared with normal pancreatic tissue (n = 20). Using quantitative reverse transcription-PCR, a significant overexpression of HO-1 mRNA was detected in all cancer samples (27 of 27). On average, a 6-fold up-regulation of HO-1 mRNA \( (P < 0.05) \) in the pancreatic cancer samples compared with the normal pancreatic tissue was observed (Fig. 1A). Western blot analysis revealed similar results on the protein level: pancreatic cancer tissue showed on average 3.5-fold higher HO-1 levels in comparison with normal pancreatic tissues \( (P < 0.05; \text{Fig. 1B}) \).

**Heme oxygenase-1 is found in tumor-associated immunocytes and pancreatic cancer cells.** Next, the localization of HO-1 was determined in normal and cancer tissues. Immunohistochemistry analysis using an αHO-1 monoclonal antibody revealed that HO-1 is located in pancreatic cancer cells as well as in immunocytes located next to the tumor mass (Fig. 1C).

Pancreatic cancer cell lines showed varying HO-1 mRNA expression levels: PANC-1 and Colo 357 cell lines had very high HO-1 expression levels, whereas MiaPaCa-2 showed low levels of HO-1 mRNA expression. In SU8686 cells HO-1 mRNA expression was not detectable (Fig. 2A and B). By confocal microscopy strong HO-1 signals were present in the cytoplasm of all Panc-1 and Colo 357 cells, whereas only some MiaPaCa-2 cells and none of the SU8686 cells showed positive staining (Fig. 2C).

**Heme oxygenase-1 can be induced in pancreatic cancer cell lines.** To determine whether HO-1 is inducible in pancreatic cancer, tumor cells were exposed to exogenous stress in vitro along with commonly used anticancer treatment modalities such as radiotherapy and chemotherapy.

When exposed to continuous oxidative stress, induced by dd-buthionine-[S,R]-sulfoximine and 1,1-dimethyl-4,4-biperidinum dichloride, all four pancreatic cancer cell lines showed a transient up-regulation of HO-1 (Fig. 3). Quantification data from the Western blot revealed that oxidative stress increased the expression of HO-1 protein in Colo 357 by 1.2 times, in Panc-1 by 1.5 times, in MiaPaCa-2 by 3.5 times, and in SU8686 by 8.2 times compared with the nontreated controls. Next, we treated the pancreatic cancer cells with an ECG50 dose of gemcitabine for 24 hours or a 20 Gy bolus dose of γ-radiation in vitro. Both treatments resulted in multiple fold increases of HO-1 in all the cell lines and to de novo expression in SU8686, which showed no detectable basal levels of HO-1 (Fig. 3): treatment with gemcitabine increased the expression of HO-1 in Colo 357 by 2.1 times, in Panc-1 by 1.3 times, in MiaPaCa-2 by 1.3, and in SU8686 by 9.6 times, compared with the nontreated controls. The 20 Gy bolus dose of γ-radiation resulted in similar changes in the expression of HO-1, with 2.9-fold increase in Colo 357, 1.3-fold increase in Panc-1, and 8.8-fold increase in SU8686. There was no significant induction of HO-1 observed in the MiaPaCa-2 cell line.

**Targeted inhibition of heme oxygenase-1 activity results in diminished pancreatic cancer cell proliferation.** To determine whether the induced high expression levels of HO-1 are responsible for the high proliferation index of pancreatic cancer cells, we suppressed HO-1 expression through siRNA transfection of a 21-nucleotide duplex HO-1 siRNA and evaluated the growth behavior of the cancer cells. The Silencer Negative Control siRNA 1 was used as a negative control. 48 hours post-transfection, Western blot analysis revealed a significant decrease in HO-1 levels in all tested cancer cell lines, compared with the negative control (Fig. 4A): in Colo 357, siRNA transfection down-regulated the HO-1 protein level by 3.5 times compared with the control. In Panc-1, the HO-1 level was 3.8 times lower after siRNA transfection. In MiaPaCa-2 and SU8686 HO-1 levels were 2.3 and 1.6 times lower than in the nontreated controls. Furthermore, the activity of HO-1 was decreased accordingly in all four cell lines: in Colo 357, siRNA transfection decreased bilirubin level by 33 ± 2%.
compared with the control; in Panc-1, bilirubin level was 32 ± 6% lower after siRNA transfection, in MiaPaCa-2 and SU8686, it was 21 ± 4% and 36 ± 2%, respectively, lower than in control group (P < 0.037; Fig. 4B).

Subsequently, the proliferation rates of the pancreatic cancer cells were evaluated at different time points by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Down-regulation of HO-1 activity was associated with a significant inhibition of the proliferation rate in all four pancreatic cancer cell lines (Fig. 5). The proliferation decreased in comparison with the controls in Panc-1 by 24 ± 2%, in MiaPaCa-2 by 24 ± 19, in Colo 357 by 25 ± 6%, and in SU8686 by 18 ± 5%. The evaluation of cell viability at 72 hours revealed that at least partly this effect is based on a diminished survival of the cancer cells; decrease of viability in comparison with controls: Panc-1 by 13 ± 7, MiaPaCa-2 by 12 ± 3%, Colo 357 by 8 ± 5%, and SU8686 by 7 ± 6% (all P < 0.05; Fig. 5). In contrast, there was no statistically significant difference between native cells and cells transfected with control siRNA (data not shown).

**Targeted inhibition of heme oxygenase-1 sensitizes pancreatic cancer cells to oxidative stress, radiotherapy, and chemotherapy.** The anti-inflammatory and antiapoptotic activities of HO-1 help cells to adapt to stress and injury. To determine whether the radioresistance and chemoresistance of pancreatic cancer cells are due to high levels of HO-1 expression, we exposed cancer cells with inhibited HO-1 activity to oxidative stress as well as radiotherapy and chemotherapy.
Forty-eight hours after transfection of HO-1 siRNA, the pancreatic cancer cells were exposed to dd-buthionine-[S,R]-sulfoximine and 1,1′-dimethyl-4,4′-biperidinum dichloride (which mediates oxidative stress), gemcitabine (EC_{50} dose), or γ-radiation (20 Gy). Seventy-two hours later, cell viability was evaluated. HO-1 expression and activity was still suppressed at this time. The inhibition of HO-1 sensitizes pancreatic cancer not only to oxidative stress but also to radiotherapy and chemotherapy. We found that decreased expression and activity of HO-1 resulted in increased cell sensitivity to oxidative stress and decreased the numbers of viable cells by 12% to 20% (P < 0.05; Fig. 6A). A 5% to 9% reduction in viability was observed after HO-1 inhibition and chemotherapy with gemcitabine (P < 0.05; Fig. 6B). Finally, a significant sensitization effect of HO-1 inhibition was also observed towards radiotherapy (P < 0.05; Fig. 6C), with an overall reduction of 13% to 23% in cell viability.

Discussion

A wide range of stress stimuli in all cells induces HO-1. In several experimental systems, it was shown that the induction of HO-1 is a fundamental self-defense process of cells to assaults from the environment (35). The anti-inflammatory and antiapoptotic effects of HO-1 and its metabolites protect cells, tissues, and even whole organs. Furthermore, HO-1
influences cell growth in a specific manner (36). Based on these observations, several new experimental treatment options are under development for various inflammatory disorders, transplant rejection, endotoxic shock, atherosclerosis, and respiratory diseases (37, 38). HO-1 induction inhibits the inflammatory reactions or even prevents or ameliorates the course of the disease (35).

Different human cancers express high levels of HO-1, which may provide them a growth advantage (39). Therefore, inhibition of HO-1 might lead to reduced tumor growth in vitro and in vivo (11, 28, 30).

The data presented here show that HO-1 expression is significantly up-regulated in human pancreatic cancer compared with normal pancreatic tissue. A strong overexpression was found in cancer cells but also in immunocytes surrounding the cancer cells.

In human gliomas and melanomas, for example, only tumor-associated macrophages were expressing HO-1 (26, 27, 40, 41), whereas in human prostate, esophageal, tongue, and renal cancer only the tumor cells revealed strong HO-1 signals (23, 24, 42, 43). Expression of HO-1 in macrophages within the tumor seems to correlate with increasing vascular density and therefore ongoing neovascularization, which is known to promote local tumor growth (26, 27, 40, 41). In vitro and in vivo studies in a pancreatic cancer model showed that HO-1 stimulates angiogenesis and increases endothelial cell survival (28, 44–47). Furthermore, in melanoma and oligodendrogliaoma, expression levels of HO-1 in macrophages correlate with tumor cell invasiveness and poor prognosis (27, 40). These data imply that HO-1 plays a role in tumor angiogenesis mediated by activated macrophages and also mediates anti-apoptotic effects on endothelial cells.

The strong HO-1 positivity in pancreatic tumor cells in situ and in several pancreatic cancer cell lines points to yet another role of HO-1 in tumor pathogenesis. The expression level varied among cancer cells and also in different cell lines. Direct correlation of the expression level with clinical or histomorphologic data could, however, not be established in this study. In contrast, others observed some prognostic value of HO-1 expression in tumors, although with contradictory results:

Fig. 3. Adjuvant treatment leads to HO-1 induction. Western blot shows that 24 hours of exposure to oxidative stress (induced by 100 mmol/L dithio-bis(2-nitrobenzoic acid) (S,N′)-sulfonamide and 100 mmol/L 1,1-dimethyl-4,4-biperidinum dichloride) treatment of the pancreatic cancer cells with EC50 dose of gemcitabine for 24 hours, and 20 Gy bolus dose of γ-radiation resulted in several fold increase of HO-1 in all the cell lines and it was also detectable in SU8686 compared with the control.

Fig. 4. Target siRNA down-regulates HO-1 expression in cancer cells. Western blots with a HO-1 mouse monoclonal antibody (1:200) and densitometric quantification of these blots showed that the transfection of siRNA for HO-1 mRNA (siRNA) results in a several fold decrease of HO-1 expression compared with the negative control siRNA transfection (A). In Colo 357, siRNA transfection down-regulated HO-1 by 3.5 times compared with the control. In Panc-1, HO-1 level was 3.8 times lower after siRNA transfection. In MiaPaCa-2 and SU.86.86, it was, respectively, 2.3 and 1.6 times lower than in the control group. Bilirubin level measurements in cell growth medium showed that that the transfection of siRNA for HO-1 mRNA (siRNA) results in a decrease of bilirubin production compared with the negative control siRNA transfection. This further confirms our notion that the expression and functional activity of HO-1 can be successfully down-regulated by means of specific siRNA interference. In Colo 357, siRNA transfection decreased bilirubin levels by 33 ± 2% compared with the control; in Panc-1, bilirubin level was 32 ± 6% lower after siRNA transfection; in MiaPaCa-2 and SU.86.86, it was, respectively, 29 ± 4% and 36 ± 2% lower than in the control group (*, P < 0.037; B).
Yanagawa et al. reported that low level HO-1 is associated with an increased risk of developing lymph node metastasis in tongue squamous cell carcinoma (42). On the other hand, in prostate and melanoma cancers, increasing HO-1 expression was associated with progression (24, 40). Further studies using higher numbers of human pancreatic tumor samples and comparison with inflammatory diseases of the pancreas will be necessary to illustrate the potential prognostic and diagnostic role of HO-1 in pancreatic carcinoma.

Here we show that HO-1 expression is directly associated with the proliferation and/or survival of pancreatic cancer cells. Specific inhibition of HO-1 expression and activity by siRNA transfection leads to diminished proliferation, which is only partly based on cytotoxicity. As the specific inhibition of HO-1 has only modest effect on the viability of the pancreatic cancer cells, the clearly decreased proliferation rate may be based on direct effect on the cell cycle.

It seems that HO-1 also acts as an endogenous protection mechanism in pancreatic cancer cells, which potentially makes

**Fig. 5.** Down-regulation of HO-1 leads to growth inhibition of pancreatic cancer cells. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay shows that down-regulation of HO-1 with siRNA leads to the statistically significant growth inhibition of pancreatic cancer cell lines Colo 357 with \( P < 0.04 \), Panc-1 with \( P < 0.03 \), MiaPaCa-2 with \( P < 0.03 \), and SU.86.86 with \( P < 0.04 \), compared with the control group, that was equal to 100% at every time point and corresponding values of the siRNA group were calculated based on this condition. Columns, results of FACS/propidium iodide analysis 72 hours post-transfection, which show that the number of viable cells statistically significantly decreases in siRNA group compared with the control group.

**Fig. 6.** Down-regulation of HO-1 expression sensitizes cancer cells to anticancer therapy. Our data shows that the down-regulation of HO-1 with siRNA sensitizes the pancreatic cancer cells to adjuvant treatment. FACS analysis with propidium iodide staining proves that down-regulation of HO-1 lowers the number of viable cells further by 12% to 20% (\( *, P < 0.05 \)) after exposure to oxidative stress in comparison with the control group (A). Decreased expression and activity of HO-1 also results in increased cell sensitivity to gemcitabine and decreased number of viable cells by 5% to 8% (\( **, P < 0.05 \)), as shown by FACS analysis (B). FACS analysis also confirms that down-regulation of HO-1 sensitizes the pancreatic cancer cells to radiotherapy, thus resulting in a decreased number of viable cells by 13% to 23% (\( ***, P < 0.05 \)) after this treatment (C).
cells more resistant to oxidative stress and apoptotic stimuli, thus providing growth advantage. Moreover, the HO-1 system is known to have direct effects on various components of the cell cycle machinery, which leads to cell-specific promotion or inhibition of cell proliferation depending on the cell type (36). Because tumor growth is based on augmented cell growth and prolonged cell survival (based on resistance to apoptosis), it is possible that HO-1 develops its tumor-promoting activity by increasing cell proliferation and protection towards apoptosis.

More importantly, we show that HO-1 may represent mechanism for pancreatic cancer cells towards anticancer drugs and radiotherapy. The destructive mechanisms of radiotherapy as well as chemotherapy on cancer cells are mainly based on the generation of oxidative stress and/or induction of apoptosis (48). In our present, study we show that radiation as well as gemcitabine (both widely used therapeutic approaches for pancreatic cancer today) lead to the induction of HO-1 in pancreatic cancer cells. This underlines the role of HO-1 as a stress response gene, showing pronounced up-regulation after first time treatment, leading to the resistance of further therapy, a generally known clinical problem in cancer treatment. That inhibition of HO-1 expression and activity in pancreatic cancer cells having high constitutive HO-1 levels enhances their response to radiotherapeutic and chemotherapeutic supports this view. Recent studies in other models made similar observations: induction of HO-1 made leukemia and colon cancer cells resistant to merocyanine and pyrrolidine dithiocarbamate, two experimentally used chemotherapeutic drugs (49, 50). In an experimental murine sarcoma model pretreatment with the HO-1 inhibitor PEG-ZnPP augmented the cytotoxic effect of several chemotherapeutics (51).

The molecular basis of these effects on cancers is not yet clear. From other cells and experimental systems, one might hypothesize that three processes induce resistance of cancer cells: (I) HO-1 diminishes the pro-oxidant pool intracellularly by degrading heme, increasing cellular iron efflux and inducing the synthesis of ferritin, a strong anti-oxidant; (II) HO-1 metabolites CO and biliverdin/bilirubin revealed antiapoptotic and inflammatory effects; (III) through direct protein-protein interaction, HO-1 may have further enzyme-independent protective functions (35, 52). Furthermore, HO-1 does also directly influence several cell signaling pathway, such as mitogen-activated protein kinases and prostaglandins, which are known to have crucial roles in pancreatic cancer pathogenesis (53). Most probably, ROS play a crucial role, as we show that pure oxidative stress is significantly more effective in killing pancreatic cancer cells after HO-1 inhibition. Irradiation revealed similar enhancement of killing of pancreatic cancer cells, whereas the alteration of the gemcitabine effects was weak to modest. Other HO-1- and ROS-independent pathways may be in case of gemcitabine of more importance.

In future studies, the known pathways have to be evaluated in cancer models both in vitro and in vivo. This will help us gain a better understanding of the treatment resistance processes, especially in pancreatic cancer. We believe further that specific tumor-targeted or even systemic inhibition of HO-1 or its metabolites in vivo could be of great therapeutic benefit because it could sensitize tumor cells to the oxidative burst from inflammatory cells (54, 55). It may also enhance the inflammatory response in tumor tissue and reinforce the host defense system by increasing tumor necrosis factor-α production in macrophages, as some authors suggest that CO derived from heme degradation could potentially inhibit the production of this proinflammatory cytokines in activated macrophages (20, 56).

In conclusion, high HO-1 levels in pancreatic cancer cells may, at least partly, be responsible for their resistance to anticancer therapy. Furthermore, high HO-1 expression seen in human pancreatic cancer may act as endogenous protection mechanism, which is initiated during carcinogenesis to make pancreatic cancer cells resistant to environmental stress such as immune attack, hypoxia, and nutrition deprivation. A better understanding of the exact molecular processes of the HO-1 system in pancreatic carcinogenesis may lead to new therapeutic targets. Perhaps, more important, specific inhibition of HO-1 activity in combination with conventional radiotherapy and chemotherapy may open up new perspectives in the treatment of pancreatic cancer.

References


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