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Heme oxygenase-1 accelerates tumor angiogenesis of human pancreatic cancer

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Abstract

Angiogenesis is necessary for the continued growth of solid tumors, invasion and metastasis. Several studies clearly showed that heme oxygenase-1 (HO-1) plays an important role in angiogenesis. In this study, we used the vital microscope system, transparent skinfold model, lung colonization model and transduced pancreatic cancer cell line (Panc-1)/human heme oxygenase-1 (hHO-1) cells, to precisely analyze, for the first time, the effect of hHO-1 gene on tumor growth, angiogenesis and metastasis. Our results revealed that HO-1 stimulates angiogenesis of pancreatic carcinoma in severe combined immune deficient mice. Overexpression of human hHO-1 after its retroviral transfer into Panc-1 cells did not interfere with tumor growth *in vitro*. While *in vivo* the development of tumors was accelerated upon transfection with hHO-1. On the other hand, inhibition of heme oxygenase (HO) activity by stannous mesoporphyrin was able transiently to delay tumor growth in a dose dependent manner. Tumor angiogenesis was markedly increased in Panc-1/hHO-1 compared to mock transfected and wild type. Lectin staining and Ki-67 proliferation index confirmed these results. In addition hHO-1 stimulated *in vitro* tumor angiogenesis and increased endothelial cell survival. In a lung colonization model, overexpression of hHO-1 increased the occurrence of metastasis, while inhibition of HO activity by stannous mesoporphyrin completely inhibited the occurrence of metastasis. In conclusion, overexpression of HO-1 genes potentiates pancreatic cancer aggressiveness, by increasing tumor growth, angiogenesis and metastasis and that the inhibition of the HO system may be of useful benefit for the future treatment of the disease.

Abbreviations: hHO-1 – human heme oxygenase-1; Panc-1 – pancreatic cancer cell line; SCID – severe combined immune deficient mice

Introduction

Angiogenesis, the process by which new blood vessels are formed from pre-existing ones [1, 2], has a fundamental role during embryogenesis, wound healing, reproductive functions and in some pathological conditions, such as rheumatoid arthritis, arteriosclerosis and proliferative diabetic retinopathy [3–5]. Tumors require angiogenesis for its growth and metastasis [6–8]. There are two phases in tumor neovascularization, the angiogenic switch or the pre-vascular phase and the vascular phase [6]. Tumor cells that undergo the phenotypic switch together with infiltrating cells such as fibroblasts and macrophages are able to induce phenotypic changes in endothelial cells, leading to angiogenesis [5, 9]. Endogenous angiogenic factors as well as anti-angiogenic factors released by tumor cells are though to regulate tumor angiogenesis [6].

The heme oxygenase (HO) system controls the rate-limiting step in heme catabolism [10]. To date, three HO isoforms (HO-1, HO-2 and HO-3) have been identified that catalyze this reaction [11–13]. HO-1 is a 32 kDa heat shock protein [14–16] which is inducible by numerous noxious stimuli [17–22]. HO-2 is a constitutively synthesized 36 kDa protein which is abundant in brain and testis [11]. HO-3 is related to HO-2 but is the product of a different gene, and its ability to catalyze heme degradation is much less than that of HO-1 [23]. HO-1 was found to play a role in stimulation of angiogenesis,

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Deramaudt et al. [24] showed that overexpression of HO-1 gene in endothelial cells caused a significant increase in angiogenesis. At the same time, upregulation of HO-1 gene was observed by several angiogenic stimulating factors, such as interleukin-1 and 6 (IL-1, Il-6), tumor necrosis factor (TNF) and transforming growth factor-β (TGF-β) [20, 25]. These evidences suggest that HO-1 enzyme could cause exacerbation and stimulation of tumor growth by increase vasculature of the tumor. A relation between malignant behavior and alteration of HO may exist. Elevated HO activity was found in renal adenocarcinoma, compared with juxtatumor or normal renal tissues and this elevation was attributed solely to HO-1 gene expression [26]. In addition, increased expression of HO-1 was detected in lymphosarcoma [27], benign prostatic hyperplesia and prostate cancer [28], acute hepatitis and hepatoma stage [29]. In human gliomas, HO-1 may be a useful marker for macrophage infiltration as well as neovascularization [30]. Pancreatic cancer has a very poor prognosis and high mortality rate [31, 32]. Available chemotherapy regimen are not consistently effective in patient with advanced pancreatic cancer and novel methods which take advantage of molecular mechanisms in cancer, are needed to improve the overall outlook for these patients [33].

The objective of this study was to examine the effect of modulation of human HO-1 gene on angiogenesis, tumor growth and metastasis of pancreatic carcinoma in severe combined immune deficient (SCID) mice. Our data demonstrated for the first time that overexpression of human HO-1 accelerates tumor growth, stimulates early stages of angiogenesis and increases the occurrence of lung metastasis. Inhibition of HO system by using SnMP, is of benefit effect in controlling tumor growth, spread and angiogenesis.

Materials and methods

Cell culture conditions

Pancreatic cancer cell line (Panc-1) human pancreatic cancer cell line were cultured in RPMI 1640 (GIBCO/BRL, Grand Island, New York) supplemented with 10% FCS, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Human umbilical vein endothelial cell line (HUVEC) were obtained from (Clonetics, Walkersville, Maryland) and grown in complete EGM medium (Clonetics) and used for the *in vitro* experiments. All cells were incubated at 37 °C in a 5% CO_2/95% air chamber.

Reagents and antibodies

Tin (stannous) mesoporphyrin (SnMP) (Porphyrin Products, Inc., Logan, Utah) was used as an inhibitor of HO activity [34]. Rabbit anti-asialo GM-1 antibody (Wako, Japan) was administrated via tail vein (100 μl/mouse) twice a week, the first time 3 days before the tumor cells inoculation.

Mice

Male, 6–8-week-old SCID mice (Fox Chase C.B-17/Icr-SCID Jcl), were kept in pathogen-limited environment and allowed free access to food and water.

Transduction of Panc-1 cells with hHO-1 gene

Replication-deficient retrovirus vector encoding human HO-1 and neomycin resistance gene, neo^r, (LSN-hHO-1) or the neo^r gene alone (LXSN) were constructed in our laboratory as described by Quan et al. [35]. The clones of packaging cell line PA317/LSN-hHO-1 and PA317/LXSN with viral titers of about 1.5×10^7 colony forming unit (cfu)/ml were used in the experiment below as described before [35]. By using the supernatants of the above retroviral packaging cells, we infected Panc-1 cells. Infected cells were selected for neo^r in a medium containing G418 (600 μg/ml); (GIBCO, NY) as described by Yang et al. [36]. After selection, stable transduced tumor cells lines Panc-1/hHO-1 and Panc-1/mock were obtained.

Detection of neo^r gene and hHO-1 mRNA by PCR reaction

In order to confirm hHO-1 expression in tumor and endothelial cells. Total RNA was extracted from Panc-1, Panc-1/hHO-1 and Panc-1/mock and HUVEC using Rneasy Mini Kit (Qiagen KK, Tokyo, Japan). RT was carried out by using the advantage RT-kit (Clontech). For detection of integration of neo^r in Panc-1/mock and Panc-1/hHO-1, Genomic DNA was extracted using High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Indianapolis, Indiana). Hundred ng of the extracted DNA and cDNA was amplified using specific primers for amplifying hHO-1, neo^r (Table 1). Cycling parameters for amplification were: 95 °C, 1 min; 60 °C, 1 min; 72 °C, 1–3 min, for 30 cycles, and extended at 72 °C for another 5 min. G3PDH amplification was used as an internal control. Ten μl aliquots from the amplification reactions were electrophoresed on 1.2% agarose and visualized by Ethidium bromide staining under UV light.

Western blot analysis of hHO-1

Cell lysate from Panc-1, Panc-1/hHO-1 and Panc-1/mock was used for measurement of HO-1 protein as

Table 1. Primers used in PCR reactions.

Product	Primers	bp
hHO-1	CAGGCAGAGAATGCTGAGTT GATGTTGAGCAGGAACGCAGT	557
neo^r	AAGATGGATTGCACGCAGG GCAAGGTGAGATGACAGGAG	313
G3PDH	GCCACATCGCTAAGACACCATGGG CCTGGTGACCAGGCGCCCAAT	81

previously described [36]. Briefly, 30 μg of lysate supernatant was separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Amersham, Piscataway, New Jersey) using a semidry transfer apparatus (Bio-Rad). The membranes were incubated with 5% milk in 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, and 0.05% Tween 20 (TBST) buffer at 4 °C overnight. After washing with TBST, the membranes were incubated with a 1:2000 dilution of anti-hHO-1 antibodies for 1 h at room temperature with constant shaking. The filters were then washed and subsequently probed with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham) at a dilution of 1:2000. Chemiluminescence detection was performed with the Amersham ECL detection kit according to the manufacturer's instructions.

Subcutaneous inoculation of tumor cells and in vivo *assessment of tumor growth*

Mice were divided into four groups and 100 μl of sterile PBS containing 1.2×10^6 cells, viability 98%, of either Panc-1 ($n = 4$), Panc-1/mock ($n = 4$) or Panc-1/hHO-1 cells ($n = 12$) were inoculated subcutaneously. One-third of the mice from the later group were administered weekly with 50 μl of aqueous solution of SnMP (15 μmol/kg body weight) and another third received a double dose of SnMP (30 μmol/kg body weight) weekly. Using a caliper, the diameters of the tumors were measured and the tumor volume was determined twice a week, in a blind fashion by the formula: $V = D \times d^2 \times 0.4$, where V is the tumor volume, D, the biggest dimension, and d, the smallest dimension.

In vitro *angiogenesis in co-culture of tumor cells with endothelial cells*

A growth factor-induced basement membrane Matrigel matrix (BD Bioscience, Bedford, Massachusetts) was used for assessment of *in vitro* capillary formation, as previously described [24]. The Matrigel matrix (10 mg/ml) was thawed, mixed to homogeneity and transferred, using cooled pipettes, to 12-well plates, which were incubated at 37 °C to induce gelling. Panc-1, Panc-/hHO-1 and Panc-1/mock were suspended in RPMI 1640 medium. Also these cells were co-cultured with endothelial cells HUVEC. Cells were diluted with 1% FBS to a final concentration of 50,000 cells/ml in MCDB 131 medium (GIBCO) and were pipetted onto the 1:2 Matrigel matrix. Then cultures were photographed.

Transparent chamber model

The two titanium frames that make the framework of transparent chamber were designed in our laboratory, manufactured by Aoba Science Ltd (Sendai, Japan) and implanted as previously described [37]. After implantation, the skinfold chamber bearing mice were housed individually and allowed to recover three days.

Panc-1, Panc-1/HO-1 or Panc-1/mock (1×10^6 cells) was implanted on the skin muscular layer. Tumor angiogenesis observation and off-line analysis were performed as described by Lozonschi et al. [38].

Lung colonization model

Panc-1 cells, Panc-1/mock, Panc-1/hHO-1 and PK-9 cells were prepared separately as a single cell suspension in 100 μl sterile PBS at a concentration of 2×10^6 cells/ml, and used for i.v. injection in lateral tail vein as follow: animal was divided into five groups, each group contain six mice, first group was injected with Panc-1 cells the second with Panc-1/mock, the third group was injected with Panc-1/hHO-1, the fourth group was injected with PK-9 cells, which is not expressing hHO-1, and the last group was injected with Panc-1/hHO-1 and then injected with SnMP (30 μmol/kg/week, i.p.). After 20 days, animals were sacrificed. The NIH instruction for use of experimental animals were followed during the experiment.

Immunohistochemical staining of tumor sections

Tumor specimens were fixed in 10% formalin and then embedded in paraffin. The lectin staining of endothelial cells was performed on 3–5 μm sections of paraffin-embedded samples using biotinylated wheat germ agglutinin-WGA-1 (Vector Laboratories, Burlingame, California) as previously described [39]. The proliferation index was assessed by staining for human Ki-67, an antigen expressed throughout the cell proliferation cycle but absent in quiescent (G_0) cells. The staining protocol using mouse anti-Ki-67 monoclonal antibody- MIB-1 (Immunotech, Marseille, France) was done as previously described [40]. Sections from lymph nodes with reactive germinal centers were used as positive controls. Quantification of the staining results was made by determining the number of stained cells in at least 10 high power fields.

Statistical analysis

All experiments were performed in duplicate. Statistical analysis of data was done by one-way analysis of variance (ANOVA) and Student's t-test. $P \leq 0.05$ was considered significant.

Results

Detection of neo^r gene and human HO-1 mRNA by PCR reaction

To evaluate the integration of retrovirus in Panc-1 and Panc-1/mock. Cellular genomic DNA neo^r fragment

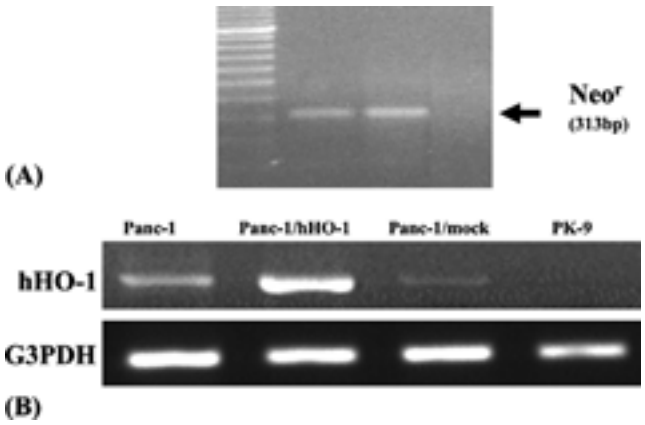

Figure 1. PCR of neo^r gene and human HO-1 cDNA. To assess the integration of recombinant deficient retrovirus in tumor cells (A). Neo^r gene fragments, from Panc-1 infected with LXSN retrovirus (Panc-1/mock), lane 1; cells infected with LSN-hHO-1 (Panc-1/hHO-1), lane 2; and non-infected Panc-1 cells, lane 3; were amplified by PCR, electrophoresed on 1.2% agarose gel. RT/PCR of human HO-1 in transduced tumor cells (B), lanes 1–3, Panc-1 cells, Panc-1/mock and Panc-1/hHO-1 respectively. G3PDH amplification was used as internal control.

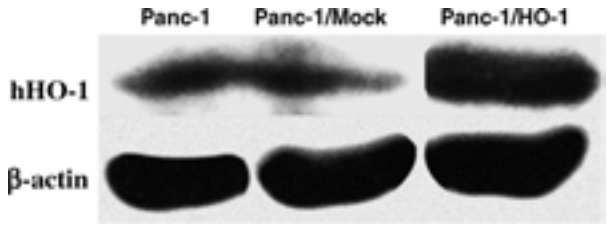

Figure 2. Western blot analysis of hHO-1 expression in human pancreatic cancer cell line (Panc-1), transduced or non-transduced with hHO-1 gene. Thirty μg of cell lysate from Panc-1, Panc-1/hHO-1 or Panc-1/mock was used for measurement of HO-1 protein by western blot technique. All cells expressed hHO-1, with Panc-1/hHO-1 cells demonstrate an increased level of hHO-1 protein (lane 3) compared to Panc-1/mock (lane 2) and non-transduced Panc-1 (lane 1). β-actin for each samples is shown in lower panel.

was amplified by PCR. As seen in Figure 1, neo^r genes were detected in both Panc-1/hHO-1 and Panc-1/mock (lanes 1 and 2) while no signal was found in non-infected Panc-1 cells (lane 3).

Then we assessed the expression of hHO-1 mRNA in non-infected Panc-1 cells as well as cells infected with retrovirus (LSN-hHO-1 or LXSN) by RT-PCR. As shown in Figure 1, Panc-1 cells infected with LSN-hHO-1 retrovirus expressed hHO-1 at a high level (lane 2); compared to non-infected Panc-1 cells and cells infected with LXSN, Panc-1/mock (lanes 1, 3) which showed a very low basal level of hHO-1 mRNA. PK-9 cells shows no signal for HO-1 (lane 4).

Effect of retroviral-mediated hHO-1 gene transfer on endogenous hHO-1 protein

Cell lysate from Panc-1, Panc-1/hHO-1 or Panc-1/mock was used for measurement of HO-1 protein by western blot technique. As seen in Figure 2, all cells expressed human HO-1, with Panc-1/hHO-1 cells demonstrate an increased level of hHO-1 protein (lane 3) compared to Panc-1/mock (lane 2) and non-transduced Panc-1 (lane 1). β-actin for each samples is shown in lower panel.

Effect of hHO-1 on subcutaneous tumor growth

To study the effect of hHO-1 on tumor growth, tumor volume was measured as described in Materials and methods. As shown in Figure 3, subcutaneous tumors were developed in all groups of SCID mice. Subcutaneous tumor of Panc-1/hHO-1 implant showed 1.6-fold increase in tumor volume compared to tumor of Panc-1 implant. This accelerated growth occurred early 18 days after tumor cell inoculation. Intraperitoneal administration of SnMP (15 and 30 μmol/kg), a powerful inhibitor

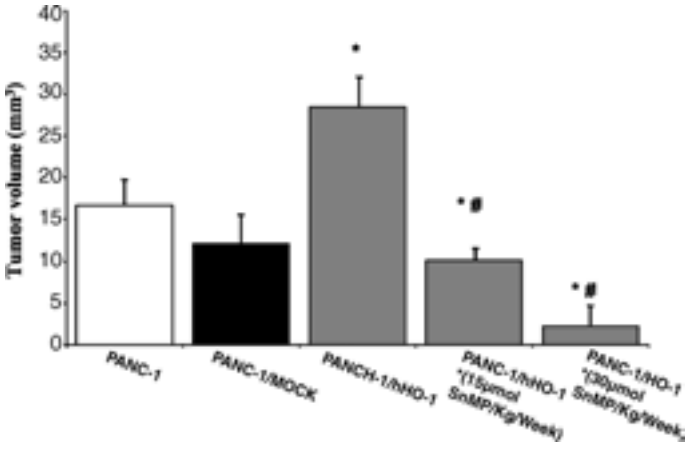

Figure 3. Effect of hHO-1 gene on tumor growth. Tumors were developed in all groups of SCID mice after inoculation of 1.2×10^6 Panc-1, Panc-1/mock or panc-1/hHO-1. Assessment of tumor volume was done. Accelerated growth was marked in Panc-1/hHO-1 group, 18 days after tumor cell inoculation. Intraperitoneal administration of SnMP (15 and 30 μmol/kg), a powerful inhibitor of HO activity, to mice with Panc-1/hHO-1 implant significantly delayed the early tumor growth in a dose dependent manner compared to tumor of Panc-1/hHO-1non-treated mice, (#$P < 0.01$) and to non-transduced Panc-1, *$P < 0.05$. Data is mean $\pm$ SD, $n = 4$ for each group.

of HO activity, to mice with Panc-1/hHO-1 implant significantly delayed the early tumor growth in a dose dependent manner compared to tumor of Panc-1/hHO-1non-treated mice, $P < 0.01$. Moreover, tumor volume in the treated mice was significantly decreased compared to that of Panc-1non-treated groups, $P < 0.05$. The most dramatic decrease in tumor volume was seen in mice injected with high dose of SnMP (30 μmol/kg) as tumor volume was 90.8 and 84.9% smaller compared to that in Panc-1/hHO-1 and Panc-1 respectively (Figure 3). However, this effect was transient, as after stop of SnMP treatment, tumor grows, eventually at a size comparable with other groups. No significant difference in tumor volume was observed between mice with Panc-1 and that with Panc-1/mock.

Analysis of tumor angiogenesis in transparent skinfold chamber

In this study, we used transparent skinfold chamber model and the *in vivo* microscopy system to assess the effect of human HO-1 on *in vivo* angiogenesis. An overview of tumor angiogenesis of Panc-1, Panc-1/hHO-1 and Panc-1/mock was determined in the implanted chambers of SCID mice at day 0 and day 11. As seen in Figure 4, at time of implantation (day 0), tumor cell masses were seen at low magnification as shaded areas. At day 11, tumors grow and appeared as reddish area with new vascular network. Marked increase in new vascular network formation was observed in Panc-1/hHO-1 tumor, (B and C), compared to Panc-1/mock tumors and Panc-1, (E, F and H, I) respectively. To assess the increase in capillary formation vascular density and mean vascular diameter were measured. As shown in Figure 5A, functional vascular density in Panc-1/hHO-1tumor was about 2-fold higher than that in Panc-1 and Panc-1/mock at day 11 ($P < 0.001$). In contrast, the mean tumor vessel diameter was 24.4% smaller in Panc-1/hHO-1 tumor compared with that of Panc-1 tumor ($P < 0.001$), Figure 5B. No significant difference in vascular density or diameter was observed between Panc-1/mock and Panc-1 tumor. This data suggest that up-regulation of human HO-1 gene accelerates angiogenesis in this model.

WGA-1 and Ki-67 immunohistochemical analysis

Staining of endothelial cells with biotinylated wheat germ agglutinin isolectin-1 (WGA-1) was applied to the paraffin-embeded sections, and was visualized histochemically by the streptavidin–alkaline phosphatase method. The binding of WGA-1 lectin was more intense

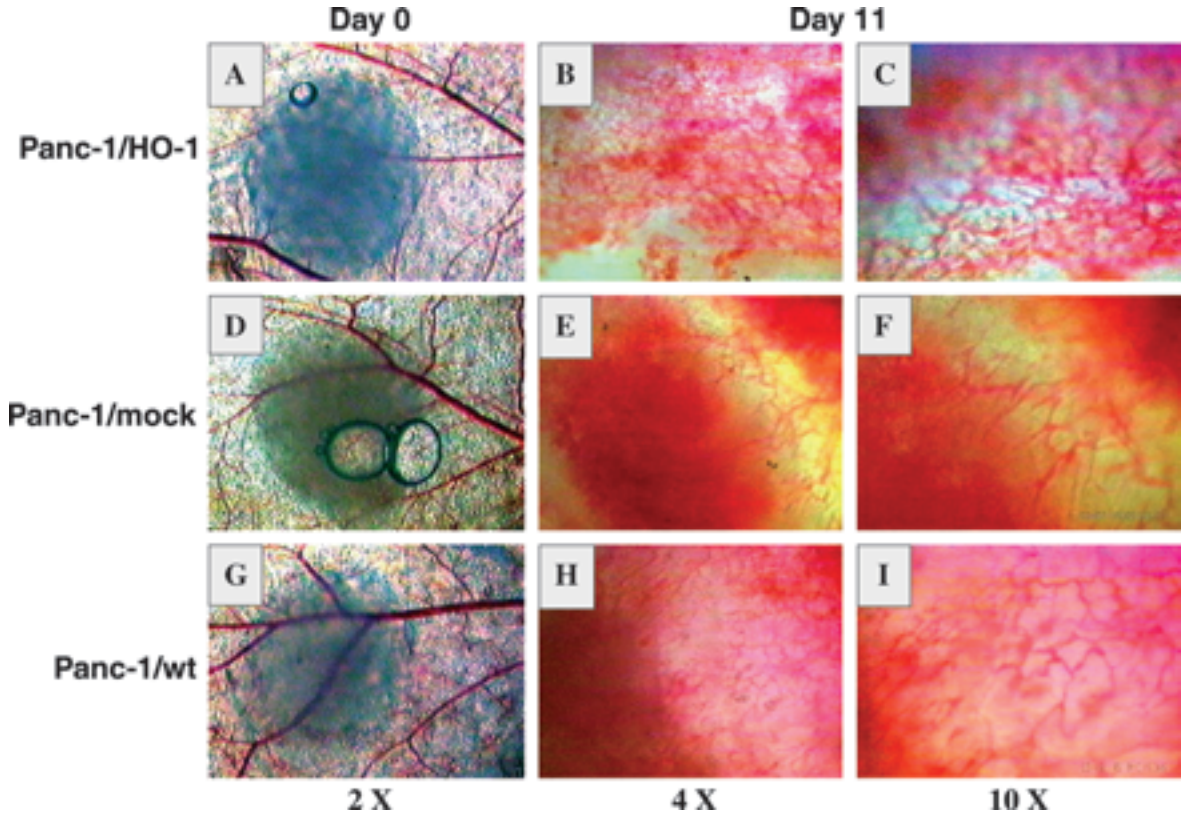

Figure 4. Tumor angiogenesis in transparent skinfold chamber. An overview of tumor angiogenesis of Panc-1, Panc-1/hHO-1 and Panc-1/mock was determined in the implanted chambers of SCID mice at day 0 and day 11. At time of implantation (day 0), tumor cell masses were seen at low magnification as shaded areas (A, D and G, ×2 objective). At day 11, tumors grow and appeared as reddish area with vascularized network. Marked increase in angiogenic vessel density was observed in Panc-1/hHO-1 tumor, (B, ×4 objective and C, ×10 objective), compared to Panc-1/mock tumors (E, ×4 objective and F, ×10 objective) or with Panc-1 (H, ×4 objective and I, ×10 objective).

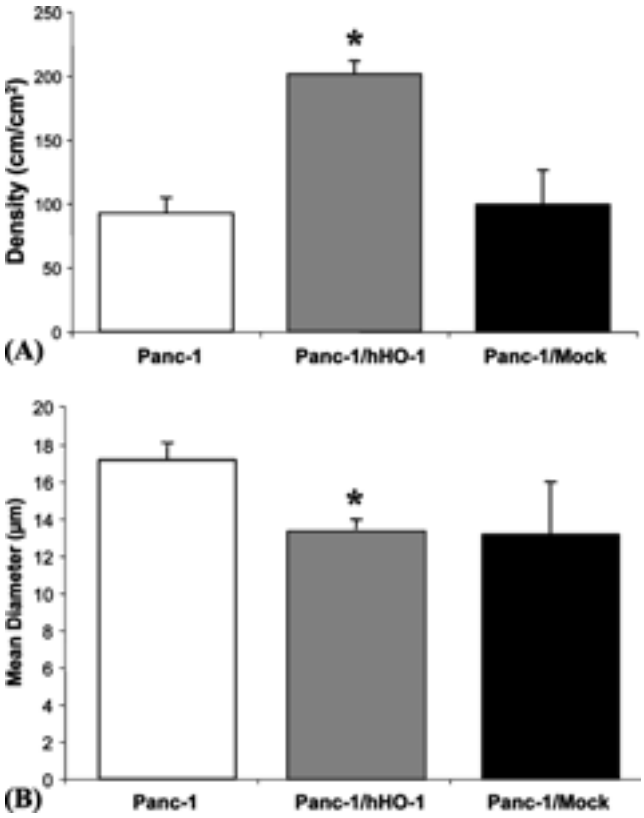

Figure 5. Vascular density and mean vascular diameter in transparent skinfold chamber model. (A) Functional vascular density in Panc-1/hHO-1tumor, Panc-1, and Panc-1/mock at day 11. (B) The mean tumor vessel diameter in the three groups. (*$P < 0.001$). No difference in vascular density or diameter was observed between Panc-1/mock and Panc-1 tumor. Data is mean ± SD.

in sections of Panc-1/hHO-1 compared to Panc-1 and Panc-1/mock sections. Statistical calculation of this results (Figure 6) showed a ($P < 0.001$) between Panc-1/hHO-1 and Panc-1 tumor section. In contrast, no significant difference in WGA-1 binding was observed between Panc-1/mock and Panc-1 sections. These results confirmed the result of skinfold chamber.

Apparently different Ki-67 immunoreactivity can be noted between Panc-1/hHO-1 cell carcinoma component and Panc-1 component (Figures 5D–F). The main value of Ki-67 proliferation index for the Panc-1/hHO-1 was 1.6-fold higher than that of Panc-1 tumor. Even though it did not reach the level of significance, $P = 0.08$, the effect of hHO-1 cannot be ignored.

In vitro *angiogenesis in co-culture of tumor cells with endothelial cells*

To confirm the effect of upregulation of hHO-1 on tumor angiogenesis, co-culture of Panc-1, Panc-1/hHO-1 or Panc-1/mock with endothelial cells in growth factor-reduced Matrigel and in low serum (1%) media was done. We found a marked and rapid development of 3-D capillary and tubular network structure only in Pac-1/hHO-1 co-culture (Figure 7). However, endothelial cells appeared elongated and in contact with each other in Panc-1 and Panc-1/mock. In addition, endothelial cell survival and proliferation were sustained by co-culture with HO-1 overexpressing tumor cells (Panc-1/hHO-1) for more than 4 weeks, compared to that of endothelial cells co-cultured with Panc-1 and Panc-1/mock (10 days).

Lung colonization model

To study the effect of overexpression of hHO-1 gene on the occurrence of metastasis, lung colonization model was used. Intravenous injection of Panc-1/hHO-1, in lateral tail vein, increased the occurrence of lung metastasis. As seen in Figure 8, large metastatic nodules was formed in the lung tissues of Panc-1/hHO-1 mice. Table 2, showed that 85% of mice with Panc-1/hHO-1 develop lung metastasis compared to only 33% in Panc-1 group. Mice survival rate was decreased in Panc-1/hHO-1 group with lung metastasis compared to the other groups (data not shown). PK-9 cells that lacks HO-1 expression did not metastasize to lungs in the 12 weeks period of this experiment Table 2. SnMP, the inhibitor of HO completely inhibited metastasis occurrence (Table 2, and Figure 8).

Discussion

Experimental evidence has directly implicated the role of HO-1 gene on angiogenesis [24, 41]. In this study, two tumor models, transparent chamber and lung colonization models, were used to detect the effect of modulation of hHO-1 gene on growth, angiogenesis and metastasis of pancreatic carcinoma.

In our previous studies, we succeed to transduce various rat and human endothelial cells with hHO-1 by using recombinant deficient retrovirus vector [35, 36]. In this study, stable transduced Panc-1/hHO-1 cells overexpressing hHO-1 gene was used for assessment of the effect of overexpression of this gene on the behavior of tumor cells.

Transparent chamber is a model particularly useful to study the temporal sequence of events occurring during early stages of angiogenesis [38]. In our study, SCID mice with Panc-1/hHO-1 inoculation, showed a marked increase in vascular network formation 11 days after tumor inoculation in skinfold chamber. Functional vascular density was higher in this group compared to control mice with Panc-1 inoculation, suggesting that overexpression of hHO-1 gene accelerate tumor angiogenesis. Increased vascular diameter is characteristic to late steps of tumor angiogenesis [38], our results showed that overexpression of hHO-1 gene did not increase tumor vessel diameter, 11 days after tumor inoculation. This finding, suggest that hHO-1 gene effects may be on the early steps of angiogenesis, at which neovascularization sprouting over vessels maturation.

Human HO-1 gene was found to have a direct effect on somatic cell growth [42]. In this study, subcutaneous

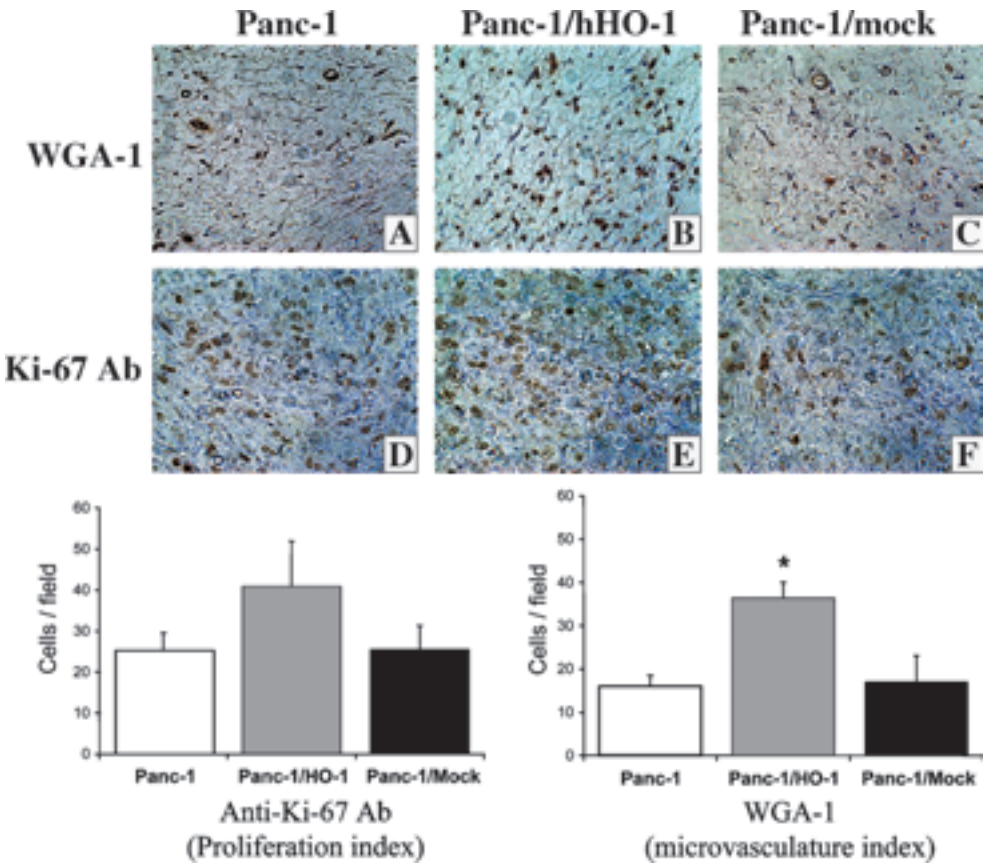

Figure 6. Immunohistochemical examination of tumor grown in SCID mice. Biotinylated wheat germ agglutinin isolectin-1 (WAG-1) staining was applied to paraffin-embedded sections and visualized histochemically by streptavidin–alkaline phosphatase method. Intense stain was seen in Panc-1/hHO-1 section (B) as compared with non-transduced Panc-1 section (A) or Panc-1/mock section (C). Proliferation index using Ki-67 staining (×10) showed marked increase in tumor proliferation rate in Panc-1/hHO-1 group (E) compared to Panc-1 group (D) or to panc-1/mock (F). Lower panel showed the statistical presentation of the immunostaining results by determining the percentage of stained cells in at least 10 high power fields. $*P < 0.01$. The results were represented as mean ± SD.

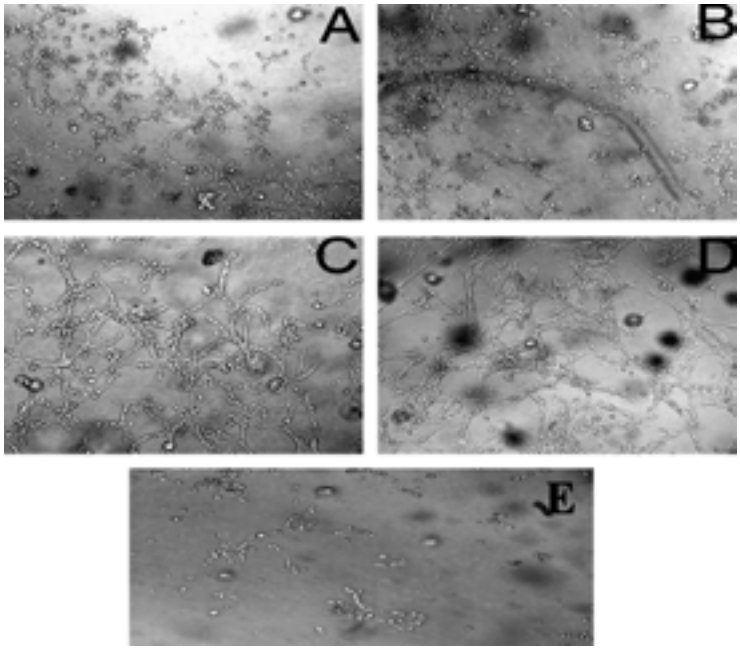

Figure 7. *In vitro* angiogenesis. Co-culture of human endothelial cells with Panc-1 tumor cells in growth factor reduced matrigel and lower serum medium. Marked and rapid development of 3-D capillary and tubular network structure in Panc-1/hHO-1 co-culture at day 7 (C) and day 21 (D) compared to Panc-1 co-culture (A), Panc-1/mock co-culture (B) at day 7 and Panc-1/mock at day 21 (E).

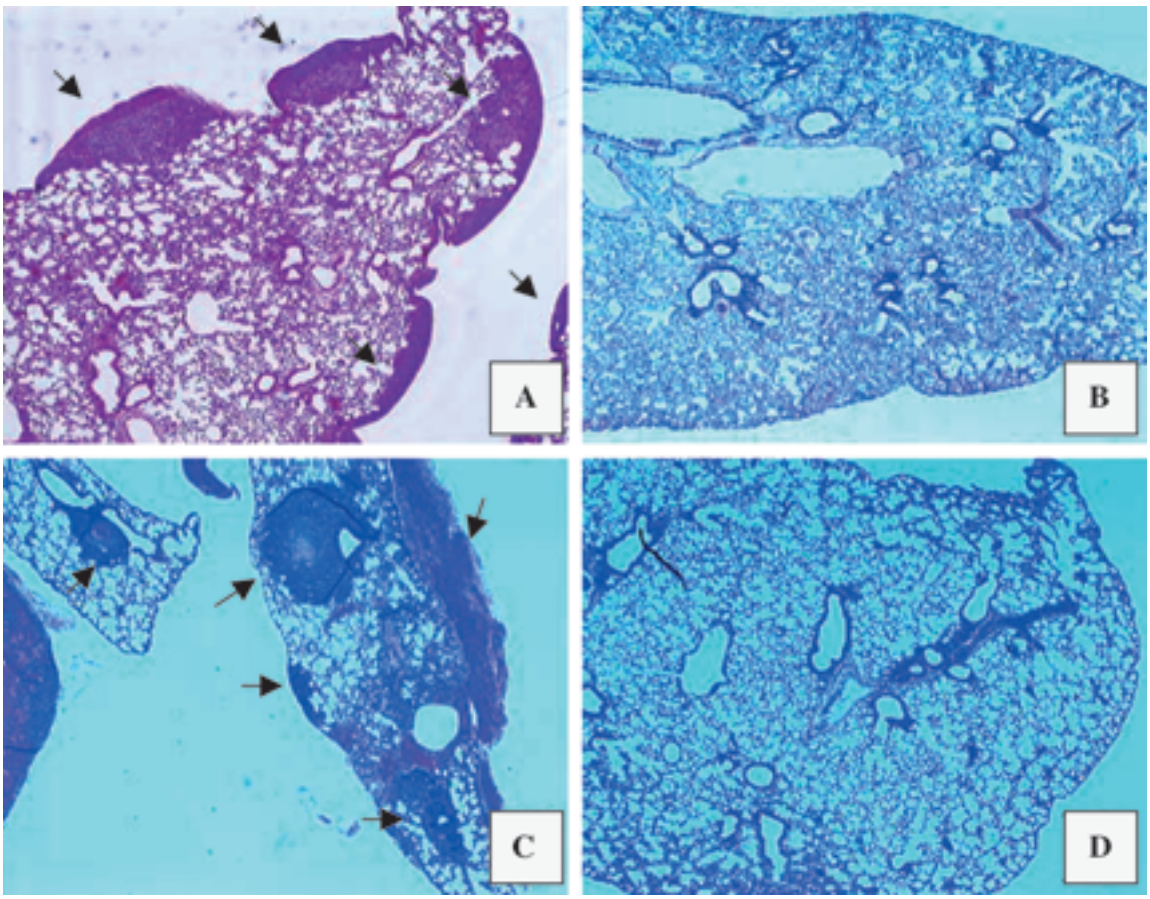

Figure 8. The effect of modulation of hHO-1 gene on the development of lung metastasis after tail vein inoculation of 2×10^6 cells in SCID mice. Large metastatic nodules are seen, by low magnification 4× objective, in Panc-1/hHO-1 (C) compared to Panc-1 tumor (A). SnMP treatment of Panc-1/hHO-1 group suppresses the formation and growth of lung metastasis (D). Inoculation of PK-9 cells, which lack HO-1 gene expression, did not show any metastasis to lung in the 20 days period of this experiment (B).

Table 2. Lung colonization model in SCID mice.

Cell line	Treatment	Mice with metastases	Percentage
Panc-1	–	2/6	33.33
Panc-1/hHO-1	–	5/6	83.33
Panc-1/hHO-1	SnMP	0/6	0
PK-9	–	0/5	0

Panc-1/hHO-1 tumor cell implant showed an accelerated early growth at day 18 with 1.6-fold increase in tumor volume compared to non-transduced Panc-1 cell implant.

The recent description of SnMP, as a powerful inhibitor of HO system, and the successful use of this molecule in treatment of neonatal jaundice [43–47] have raised interest in testing its effect on pancreatic tumor cell growth and metastasis. Our results showed that SnMP treatment of mice with Panc-1/hHO-1 tumor implant significantly delayed the early growth effect of hHO-1 in a dose dependent manner. Moreover, treatment with SnMP caused about 84.9% tumor growth retardation compared to control non-treated tumor implant. However, this effect is transient, as treated tumor grow eventually at a size comparable with that of non-treated groups after stop of SnMP treatment. This result is in agreement with our recent work on human hepatoma cell line (HepG2), which showed that SnMP markedly decreased tumor cell survival in response to oxidants [48]. The mechanism by which SnMP inhibit tumor growth was not cleared but our data suggest that it may be through limiting expansion of endothelial cells as well as cancer cells composing the tumor.

HO-1 was found to stimulate endothelial cell proliferation and accelerate cell cycle progression [49]. Our results showed that *in vitro* co-culture of Panc-1/hHO-1 tumor cells and HUVEC in growth factor reduced Matrigel and low serum medium was followed by a rapid 3-D network structure formation. On the other hand the endothelial cells appeared elongated and in contact with each other in co-culture with Panc-1 or Panc-1/mock cells. In addition HUVECs survival and proliferation were sustained by co-culture with HO-1 overexpressing cells (Panc-1/hHO-1) for more than 4 weeks, compared to 10 days survival of its co-culture with Panc-1 or Panc-1/mock cells. Previous reports showed that in 3-D culture without growth factors, HUVECs do not form tubular structure and their survival is limited to 2–3 days, when they became apoptotic [50, 51].

In our previous study, lung colonization model, was approved to be successful for determination of metastatic spread of pancreatic as well as other tumors [38]. In this study, lung metastatic model showed that overexpression of hHO-1 gene increased the occurrence of metastasis from 33.33% in non-transduced Panc-1 tumor to 83.33% in Panc-1/hHO-1. Inhibition of HO system by SnMP rendered lungs of normal weight and markedly inhibited the number of surface metastasis. Thus SnMP could possibly have limited both extravasation and subsequent tumor development. In addition, PK-9 cell line that lacks hHO-1 gene expression did not metastasize to the lung during the period of the study. Taken together, this finding confirmed the powerful role of hHO-1 gene in pancreatic tumor angiogenesis and metastasis.

In summary our study showed for the first time that hHO-1 gene stimulate the growth, angiogenesis and metastasis of pancreatic carcinoma and that blocking of HO-1 may open a new era in future control of this disease.

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