Heme oxygenase-1: a molecular brake on hepatocellular carcinoma cell migration


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Hepatocellular carcinoma (HCC) is a fatal disease with great public health impact worldwide. Heme oxygenase (HO)-1 has recently been reported as an important player in tumor angiogenesis and metastasis. However, the role of HO-1 in liver cancer metastasis is unclear. In this study, we explored genetic differences and downstream signal transduction pathways of HO-1 in liver cancer cell lines. HO-1 wild-type and mutant cell lines were generated from human liver cancer cell line HepG2. The overexpression of wild-type HO-1 decreased the migration of HepG2 cells. In contrast, the overexpression of mutant HO-1G143H increased the migration of the cancer cells. Interleukin (IL)-6 is one of the major downstream molecules that mediated this process because IL-6 expression and migration are suppressed by HO-1 and increased when HO-1 is knocked down by shRNA. In addition, we demonstrated carbon monoxide (CO) and p38MAPK are the cofactors in this signal pathway. In vivo animal model demonstrated HO-1 inhibited the tumor growth. In conclusion, in vitro and in vivo data show HO-1 inhibits the human HCC cells migration and tumor growth by suppressing the expression of IL-6. The heme degradation product CO is a cofactor in this process and inhibits p38MAPK phosphorylation.

Introduction

Liver cancer is the fifth most frequently diagnosed cancer and the second most frequent cause of cancer death in men worldwide. An estimated 748,300 new liver cancer cases and 695,900 cancer deaths occurred worldwide in 2008 (1). The high rate of recurrence and metastasis after curative resection is one of the major causes of mortality in hepatocellular carcinoma (HCC) patients. The metastasis of tumor cells is a complex multistage process involving invasion, migration, cancer cell adhesion, angiogenesis as well as the tumor cells survival and persistent growth in the distant organ (2). The study of metastasis mechanism may help identify therapeutic targets.

Heme oxygenase (HO) is the rate-limiting enzyme in heme metabolism by catalyzing degradation of the heme group into carbon monoxide (CO), free iron and biliverdin. At least three mammalian HO isoforms have been identified. HO-1, HO-2 and HO-3 and these have distinct patterns of tissue-specific gene expression. HO-1 is inducible, highly expressed in the spleen and liver, and normally found in very low levels in mammalian tissue (3,7). HO-2 is constitutively expressed in most tissues except brain and testes (4). The upregulation of HO-1 has been recognized as an adaptive response to several stress stimuli (5). There has been accumulating evidence supporting the role of HO-1 in the cytoprotection against oxidative stress and other stimuli (6). However, several studies also demonstrate that HO-1 overexpression is implicated in the pathogenesis and progression of several types of malignancies (7). In tumor-bearing mice, overexpression of HO-1 causes increased viability, proliferation and angiogenic potential of melanoma cells and augmented metastasis (8).

Recent studies have found that HO-1 is indirectly involved in metastasis and invasion of several types of malignancies, including breast cancer (9,10), prostate cancer (11,12) and lung cancer (13).

Multiple lines of evidence support the idea that biliverdin, bilirubin, free iron and CO contribute to the physiological functions of HO-1, including antioxidative, anti-inflammatory, antiproliferative and anti-apoptotic effects (6). HOs are the main producers of CO in the human body. Great attention has been recently paid to the presumed anti-inflammatory functions of CO (14). It has been demonstrated that CO at a physiological concentration (100–500 p.p.m.) inhibits the production of proinflammatory cytokines tumor necrosis factor-α and increases the synthesis of the anti-inflammatory cytokine interleukin (IL)-10 in macrophages treated with lipopolysaccharide and in vivo (15,16).

The tissue microenvironment plays an important role in tumor development and progression both at the primary site and at sites of metastases (11). Cytokines and chemokines constitute a proinflammatory network that directly contributes to malignant progression (17). IL-6, a cytokine categorized as pro- and anti-inflammatory, presents at higher levels in HCC patients than healthy individuals (18). In addition, IL-6 blood levels have been shown to be elevated in patients affected with several solid malignancies, including renal cell carcinoma (19), head and neck cancer (20) and colorectal cancer, and especially with those with liver metastases (21). Recently, Wang et al. (22) report that estrogen has the potential to inhibit lung metastasis from rat HCCs in vivo by suppression of hepatocyte growth factor and IL-6 production.

However, the role of HO-1 in the metastasis of liver cancer is still undefined. In this study, we investigated the role of HO-1 in the migration of HepG2 cells. Overexpression of HO-1 in HepG2 cells inhibited cell migration. In contrast, cells that overexpressed mutated HO-1 increased migration and IL-6 expression, which was reversed by the addition of CORM-2 or p38MAPK inhibitor (SB203580). Furthermore, we also found that HO-1 overexpression inhibited growth of HepG2 xenografts in BALB/c-nu mice.

Materials and methods

Cell culture, treatment and drug preparation

The human HepG2 hepatoma cells were maintained in Dulbecco’s modified Eagle’s medium that was supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and 100 µg/ml streptomycin at 37°C in a humidified incubator containing 5% CO2. Tricarbonyldichlororuthenium (II) dimmer (CORM-2), ruthenium (III) chloride hydrate (Ru(III)) and SB203580 (Sigma, Shanghai, China) stock solutions were prepared by dissolving in dimethyl sulfoxide at a concentration of 20 mM of stock solution.

Cell transfection

HepG2 cells were seeded at a density of 2 × 105 cells in a six-well plate and grown to 60--70% confluency in growth media. Cells were transfected with pcDNA3.1(+) containing mouse wild-type HO-1 or G143H mutant using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). The stable cell lines were selected with 500 µg/ml G418 (Sigma) and screened for HO-1 protein expression. For gene silencing, the pLL3.7, pLL3.7-HO-1shRNA1(sh1), pLL3.7-HO-1shRNA2(sh2) (4 µg) that were kindly provided by Prof Hong Zhou (Academy of Military Medical Sciences, Beijing, China) or IL-6 siRNA (100 pmol) (Genetherma, Shanghai, China) was transiently transfected into HepG2/mutHO-1G143H cells using the Lipofectamine 2000 transfection reagent (Invitrogen). Scramble siRNA served as negative control. After 24 h, the transfected cells were used for further experiments. IL-6 siRNA:
Western blot analysis
After a rinse with phosphate-buffered saline (PBS), the cells were lysed in ice for 30 min in lysis buffer (Biyuntian, Shanghai, China) containing a cocktail protease inhibitor (Roche, Shanghai, China). After centrifugation at 12 000g for 15 min, the supernatant was separated and stored at −70°C, until use. The protein concentration was determined by using a BCA protein assay kit. Protein samples were electrophoresed on a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to polyvinylidene difluoride membrane at 70 V for 3 h. The blots were incubated in fresh blocking buffer PBST (0.1% Tween-20 in PBS containing 5% non-fat dry milk, pH 7.4) for 2 h followed by incubation with the indicated antibodies in PBS with 5% non-fat dry milk for overnight at 4°C. HO-1 1:2000 (Santa Cruz Biotechnology, Santa Cruz, CA), Phospho-p38 1:1000, p38 1:1000 (Cell Signaling Technology, Danvers, MA), after washing with PBST three times, the blots were incubated with a horse-radish peroxidase-conjugated secondary antibody (1:10 000) in PBS with 5% non-fat dry milk for 2 h at room temperature. The blots were washed again three times in PBST buffer and transferred proteins were detected with an enhanced chemiluminescence detection kit (Applygen, Beijing, China).
silencing (49.1 or 33.7% inhibition for HO-1sh1 or HO-1sh2); protein levels of HO-1 were knocked down 24 h after silencing (45.8 or 16.4% inhibition for HO-1sh1 or HO-1sh2). HO-1 knockdown increased the migration of HepG2/H1B cells (Figure 2C). Together, these results demonstrate that HO-1 inhibits the migration of HepG2 cells and that this effect is correlated with HO activity.

**IL-6 is a direct effector of HO-1-regulated migration of HepG2 cells**

Maeda et al. reported that IL-6 is a critical regulator in liver metastasis(24). It is also reported that HO-1 plays an anti-inflammatory role by inhibiting the expression of IL-6 in macrophages (25). Therefore, we hypothesized that HO-1 inhibited HepG2 cell migration by inhibiting the production of the cytokine IL-6. To test this hypothesis, we assessed the effect of HO-1 on the expression of IL-6 using reverse transcription–polymerase chain reaction and ELISA. The results indicated that HO-1 overexpression suppressed the messenger RNA and protein levels of IL-6, however, overexpression of mutant HO-1G143H significantly upregulated the expression of IL-6 (Figure 3A). Silencing the expression of HO-1 significantly increased the expression of IL-6 in HepG2/H1B cells. And the effect of HO-1shRNA1 was found to be stronger than HO-1shRNA2 (Figure 3B).

To confirm that HO-1 inhibited the migration of HepG2 cells by IL-6 regulation, we silenced the expression of IL-6 in HepG2/mH1 cells. As shown in Figure 3C, the messenger RNA and protein levels of IL-6 were significantly reduced 24 h after silencing IL-6 using reverse transcription–polymerase chain reaction and ELISA. The cell migration assay suggested that IL-6 knockdown led to a decreased in HepG2 cells migration (Figure 3D). Taken together, these results demonstrate that HO-1 inhibits cell migration by inhibiting the expression of IL-6.

**CO released from HO-1-catalyzed heme degradation is involved in regulating HepG2 cell migration and the expression of IL-6**

Some groups have reported that the main function of HO-1 depends on its enzymatic products, such as CO, free iron and bilirubin (7). There is growing interest in the role of CO in the anti-inflammatory of HO-1. And our results found blood CO content of transgenic HO-1G143H mutant mice (23) was significantly reduced as compared to the littermate control (Figure 4A). Therefore, we hypothesized that the anti-migratory effects of HO-1 might be mediated by its product CO. Some groups reported a series of transition metal carbonyls, termed carbon monoxide-releasing molecules (CORMs), which mimicked some physiologic effects by induction of HO-1, which increases endogenously generated CO (26,27). To explore our hypothesis, we treated HepG2/mH1 cells with a CO-releasing molecule, CORM-2, to supply CO at various concentrations (10–100 μM) or at different times (0–24 h). The results suggested that CORM-2 supplementation in HepG2/mH1 cells inhibited the expression of IL-6 in a dose- and time-dependent manner (Figure 4C and D). At the same time, this treatment reduced their migration in a dose-dependent manner. After treatment with CORM-2 (10 and 50 μM) for 24 h, migration of HepG2/mH1 cells was significantly (P < 0.05) reduced (25 and 68.5% inhibition, respectively), as compared with the control (Figure 4E). Importantly, RuCl3 did not affect the migration demonstrating that the effect of CORM-2 was due to CO and not the presence of the ruthenium base compound (Figure 4F). These effects were not because of cytotoxicity of the compounds, as cell viability was not significantly modified by either CORM-2 (10–50 μM) or RuCl3 (20–100 μM), as determined by the MTT assay (Figure 4B). These findings suggest that the CO released from heme has a role in controlling the expression of IL-6 as well as the subsequent migration of HepG2 cells.

**Involvement of p38MAPK phosphorylation in HO-1/CO regulating the expression of IL-6 and the migration of HepG2 cells**

The p38MAPK pathway has been previously implicated in the regulation of CO-mediated anti-inflammatory signaling in macrophages (28). We hypothesized that the anti-migratory effects of HO-1/CO might be mediated via inhibition of p38MAPK phosphorylation. To test this hypothesis, we detected the level of phosphorylated p38MAPK after silencing HO-1 or HO-1G143H mutant in HepG2/H1B. As shown in Figure 5A, silencing HO-1 significantly suppressed the expression of IL-6 and the migration of HepG2 cells. We hypothesized that the anti-migratory effects of HO-1/CO might be mediated via inhibition of p38MAPK phosphorylation. To test this hypothesis, we detected the level of phosphorylated p38MAPK after silencing HO-1 or HO-1G143H mutant in HepG2/H1B. As shown in Figure 5A, silencing HO-1 significantly suppressed the expression of IL-6 and the migration of HepG2 cells.
Effect of CO on activation of p38MAPK, we treated HepG2/mH1 cells with 50 μM CORM-2 for 0.5–24 h. The results indicated that the phosphorylation p38MAPK level was decreased by treatment with 50 μM CORM-2, this effect reached a maximum at 2 h and declined thereafter (Figure 5B).

To further confirm the role of this pathway in regulation of IL-6 level and migration, we treated HepG2/mH1 cells with SB203580, an inhibitor of p38MAPK for 24 h. We found the level of IL-6 messenger RNA was dose dependently reduced after treatment with SB203580 at various concentrations (0–25 μM) (Figure 5C). The cell migration assay results indicated that 25 μM SB203580 inhibited the migration of HepG2/mH1 cells (Figure 5D). MTT assay verified that cell viability was not significant modified by 5–25 μM SB203582 (Figure 5E).

Together, these results suggest that the p38MAPK signaling pathway mediates the HO-1/CO/IL-6 pathway, which ultimately leads to the regulation of HepG2 cell migration.

**HO-1 overexpression reduced the tumor size of HepG2 xenografts**

To further determine the role of HO-1 in tumor growth and metastasis in vivo, we compared the ability of each cell line to form xenograft tumors. HepG2/mock, HepG2/H1B and HepG2/mH1 cells were injected into the left liver lobe of athymic nude male mice. Tumors developed in all injected mice. These mice were killed 7 weeks after inoculation. Metastasis to distant organs was not observed in all cases, as expected from the clinical observation that intrahepatic metastasis of human HCC occurs at a relatively late stage and less frequently than extrahepatic metastasis. As shown in Figure 6A, three groups were observed to develop multiple small liver tumors, which were clearly distinct from the principal tumor, and were occasionally located in the uninjected lobe. These lesions were considered to be intrahepatic metastatic lesions that mimicked clinical behavior. We could not calculate the numbers of intrahepatic metastasis nodules because many liver tumors had grown together. We analyzed the area of tumors using ImageJ software. The average tumor area was 1.42 ± 0.57 cm² in the control group, 0.40 ± 0.13 cm² in the HepG2/H1B-bearing mice group (P < 0.001), and 3.19 ± 0.22 cm² in the HepG2/mH1-bearing mice group (P < 0.001) (Figure 6B). Next, we performed histological analysis on the dissected tumors. These nodules were confirmed as HCC by hematoxylin and eosin staining. Hematoxylin and eosin staining revealed a few well-defined tumors and obvious necrosis (Figure 6A). In conclusion, these data suggested that expression and activity of HO-1 in HepG2 cells regulated the tumor size of HepG2 xenografts.

**Discussion**

Elevated HO-1 has been detected in several cancer cell lines and tumors, including lymphosarcoma, adenocarcinoma, hepatoma, glioblastoma,
melanoma, prostate cancers, Kaposi sarcoma, squamous carcinoma and pancreatic cancer, thereby affecting tumor cell apoptosis, proliferation, invasion and metastasis (7). Furthermore, HO-1 gene polymorphisms have been associated with an increased cancer susceptibility (29–31). However, studies regarding to the role of HO-1 in tumor progression are still controversial. Recently, the influence of HO-1 on tumor metastasis has attracted the attention of researchers. Lin et al. (9) reported that HO-1 inhibits breast cancer invasion by suppressing the expression of matrix metalloproteinase-9. Conversely, another group suggested that HO-1 promoted metastasis and invasion by upregulated matrix metalloproteinase-1 expression (10). Furthermore, Gueron et al. (11) found that overexpression of HO-1 in prostate cancer cells resulted in markedly reduced cell proliferation and migration. Even though the role of HO-1 in liver cancer cell growth was proposed, its role in HCC metastasis has yet to be elucidated. In the present study, we first discovered that the abnormal expression of HO-1 inhibits the ability of human HCC cells to migrate in vitro and in vivo.

There has been increasing evidence for a causal relationship between inflammation and cancer. Inflammation plays an important role in the tumor microenvironment (32). An increasing body of evidence indicates a key role of the pleiotropic cytokine IL-6 in the process of liver damage and carcinogenesis (24,33,34). Transfection of IL-6 into non-metastatic HCC cells makes them highly metastatic (35). HO-1 is commonly regarded as a potent anti-inflammatory enzyme. Numerous reports have demonstrated that activation of HO-1 diminishes inflammation and may result in immunosuppression. The importance of HO-1 is elegantly illustrated by studies in HO-1−/− mice, in which HO-1 deficiency results in a strongly increased generation of proinflammatory cytokines, including IL-6 (36). Therefore, we speculated that HO-1 may inhibit the migratory ability of HCC cells by regulating the level of IL-6. The results from our present study lend support to the possible involvement of HO-1 in the downregulation of IL-6, thereby conferring metastatic potential to HCC cells. Consistent with above findings, the expression of IL-6 was significantly enhanced in HepG2/mH1 cells, which obtain a high migratory ability via stably transfected mutant HO-1G143H.

Future studies are needed to elucidate the mechanism of HO-1 regulating the expression of IL-6 in HepG2 cells. Although the exact mechanisms underlying the anti-inflammatory functions of the HO-1 have not been fully elucidated, CO has been described as an efficient anti-inflammatory mediator in several models of inflammation and tissue injury. For example, in lipopolysaccharide-stimulated macrophages, CO significantly decreased the production of tumor necrosis factor and IL-6 (37). However, it is still unclear if CO is involved in HO-1 regulating the expression of IL-6 in tumor cells.

The crystal structure of the truncated rat and human HO-1 suggests that Gly139 and Gly143 interact directly with heme. Gly139His (G139H) and Gly143His (G143H) mutants of HO-1 bind heme but have no HO activity (38). Our previous studies find G139H and G143H mutants are in competition with wild-type mouse HO-1 to bind heme (23). We found that HO activity of the mouse HO-1G143H mutant transformants was reduced as compared with control
under the conditions of insufficient heme (Figure 1B). Transgenic HO-1G143H females present with anemia, enlarged spleens and iron overload. These phenotypes constitute the most obvious similarity with HO-1/C0/C0 mice (23). Therefore, in this study, we used mutant HO-1G143H overexpression to mimic HO deficiency. If HO-1G143H mimics HO deficiency, then mutant HO-1G143H overexpression will result in the reduction of the products of heme degradation, including CO. To further verify it, we detected the CO content in blood of mutant HO-1G143H transgenic mice because we cannot detect the CO content in cells. The results revealed that the CO content in blood of mutant HO-1G143H transgenic mice was reduced significantly as compared with the littermate control. Our data in vitro verified that supplementing CO rescued the enhanced cell migration and IL-6 expression by treating HepG2/mH1 cells with CORM-2. We noticed IL-6 levels appeared dip at 50 µM and then come back up at 100 µM. The CORM-2 toxicity determination demonstrated that 100 µM CORM-2 have slight cell toxicity (data not shown). Therefore, we speculated high IL-6 levels after treatment with 100 µM CORM-2 were due to cell damage followed by IL-6 releases. The use of CORM-2 in this study raises some question as to whether the effects seen are due to direct effects of CO or secondary effects of CORM-2. We excluded the effect of the ruthenium metal center using RuCl3, a CORM-2 analogue, as negative control. The data demonstrated that RuCl3 had no effects in the models studied. These results indirectly

![Fig. 4. Treatment with CORM-2 abrogates the enhanced migration and the expression of IL-6 in HepG2/mH1 cells. (A) The blood of transgenic HO-1G143H mutant mice and littermate control were collected and CO content in blood was determined by measuring the amount of HbCO formed. (B) HepG2/mH1 cells were treated with the indicated doses of CORM-2 or RuCl3 for 24 h and subjected to cell viability assay. (C and D) HepG2/mH1 cells were treated with the indicated concentrations of CORM-2 for 24 h, and the indicated times at 50 µM and messenger RNA expression of IL-6 was detected by reverse transcription–polymerase chain reaction. (E and F) HepG2/mH1 cells were treated with the indicated doses of CORM-2 or RuCl3 for 24 h and cells were allowed to migrate for 24 h toward media with 10% fetal bovine serum. Top, representative photos, bottom (graph), quantitative analyses for the cells migrating through the filter in three independent experiments. *P < 0.05, **P < 0.01.]

A molecular brake on HCC cell migration.

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suggest that CO may be responsible for the reduced migration induced by HO-1 in HepG2 cells.

CO is a secondary messenger with multiple biological activities. Depending on the cell type, CO can activate one or both of the key signaling pathways in numerous physiological and pathophysiological conditions (39,40). One of the pathways is soluble guanylate cyclase/cyclic guanosine monophosphate, which has been implicated in mediating the effects of CO on vascular contractility, the inhibition of smooth muscle proliferation (40), neurotransmission (41) and preventing apoptosis in endothelial cells (42) and fibroblasts (43). Another pathway is the p38MAPK pathway, through which CO can mediate the anti-inflammatory actions (28,43,44). Moreover, CO significantly decreased the production of IL-6 by interfering with activator protein-1 activity via a c-Jun N-terminal kinase pathway in lipopolysaccharide-stimulated macrophages (37). However, the precise mechanism of HO-1/CO regulation of migration and IL-6 gene in human HCC cells is still undefined. The results from the present study suggest that HO-1 suppresses the activation of p38MAPK. Supplementation of CO in HepG2/mH1 cells inhibited the activation of p38MAPK in a time-dependent manner. We verified that SB203580 (p38MAPK inhibitor) decreased the migration and IL-6 expression of HepG2/mH1 cells. In conclusion, HO-1/CO attenuated the expression of IL-6 and cell migration by inhibiting the activation of p38MAPK.

HCC can invade the portal vein and metastasizes to other parts of the liver even at a relatively early stage of the disease, with less tumor spread occurring outside the liver. This intrahepatic metastasis is the main cause of liver failure and death in HCC patients (45). We found the intrahepatic metastasis nodules in the liver of many mice. But we could not calculate the numbers of nodules because they had grown together. The tumor size from the three groups of mice had obvious differences. We speculated HO-1 regulated growth of liver cancer...
cells. But we cannot exclude the effect of HO-1 on intrahepatic metastasis. We hope to elucidate the effect of HO-1 on tumor growth and metastasis using other methods in the future.

In conclusion, the findings presented in this study illustrate that both in vivo and in vitro conditions, HO-1 plays an important role in HCC progression. Increasing intracellular HO-1 protein levels by transfecting cells with the HO-1 expression vector significantly inhibited the migration of HepG2 cells and led to the suppression of IL-6 and p38MAPK activation. In addition, CO reduces the migration of HCC cells by suppressing IL-6 and p38MAPK activation. These results suggest that HO-1 inducers and/or effector might play a role as a therapeutic agent in the inhibition of tumor metastasis.

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References

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