Original Research

Overexpressions of HO-1/HO-1G143H in C57/B6J mice affect melanoma B16F10 lung metastases rather than change the survival rate of mice-bearing tumours

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Abstract

Heme oxygenase-1 (HO-1) is often upregulated in tumour tissues and endows tumour cells with cytoprotection and antiapoptosis. It is worthy of note that some people show higher activity of HO-1 and some anti-cancer therapies could induce HO-1 expression in normal tissues, but the effect of HO-1 of normal tissues on tumours among these people remains unknown. To assess the effect of HO-1 of normal tissues on tumour progressiveness, we investigated the growth, metastasis and angiogenic potential of murine melanoma B16F10 cells in transgenic mice overexpressing HO-1 and its negative dominant mutant HO-1G143H, respectively. The results demonstrated that neither overexpression of HO-1 nor overexpression of HO-1G143H in normal tissues could significantly change the survival rate of tumour-bearing mice, but HO-1 overexpression could inhibit lung metastases and HO-1G143H could significantly promote lung metastases. Meanwhile, the leukocytes infiltration was reduced and angiogenesis was promoted in tumours in mice overexpressing HO-1, but the opposite was true in mice overexpressing HO-1G143H. Our findings suggested that overexpression of HO-1 might be conducive to patients bearing melanoma metastasis.

Keywords: Heme oxygenase-1, transgenic mouse, melanoma, metastasis

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Introduction

Heme oxygenases (HOs) catalyse the oxidation of heme to the biologically active products such as carbon monoxide (CO), biliverdin and ferrous iron. Three distinct mammalian HO isoforms (HO-1, HO-2 and HO-3) have been identified so far, which are the products of different genes.[1] HO-1, the inducible 32-kDa isoform and highly expressed in the liver and spleen, can be induced by oxidative stress, hypoxia, serum deprivation or toxic compounds. The enzymatic activity of HO results in decreased oxidative stress, attenuated inflammatory response and a lower rate of apoptosis. This is both due to the removal of heme, a potent pro-oxidant and pro-inflammatory agent, and the generation of biologically active products.[2]

HO-1 is very often upregulated in tumour tissues and endows tumour cells with cyto-protection and anti-apoptosis *in vitro* and *in vivo*. The upregulation of HO-1 has been shown in many tumours, such as lymphosarcoma,[3] adenocarcinoma,[4] hepatoma,[5] glioblastoma,[6] melanoma,[7] prostate cancers,[8] Kaposi sarcoma,[9] squamous carcinoma,[10] pancreatic cancer[11] and brain tumours.[12] Pharmacologic or genetic upregulation of HO-1 significantly improved survival of hepatoma,[5] melanoma,[13] thyroid carcinoma,[14] chronic myelogenous leukemia,[15] gastric cancer[16] and colon cancer cell lines.[17] HO-1 overexpression in tumour cells B16-HO-1 significantly shortened survival of mice after subcutaneous injection, and formed more metastases in lungs after intravenous injection.[13] In view of the upregulation of HO-1 in many tumours, anticancer strategies by inhibiting HO-1 were promoted. Decreased viability resulting from HO-1 inhibition was found in colon carcinoma[17] and in chronic myelogenous leukemia cells.[15] Anticancer therapies specific to HO-1 have been made *in vivo*, in tumour-bearing animals. Administration of HO-1 inhibitors zinc protoporphyrin-IX (ZnPPIX) or ZnPPIX-polyethylene glycol (ZnPPIX-PEG) resulted in a significant increase in

apoptosis of hepatoma cells in rats,[18] as well as sarcoma[19,20] and lung tumour cells[21] in mice.

Apart from much attention to the upregulation of HO-1 in tumours and anticancer therapies specific to HO-1, it is worth noting that there is difference in HO-1 expression between normal tissues. For example, short (<25 GT) repeats in HO-1 promoter results in an increased HO-1 upregulation, and chemotherapy, radiotherapy and photodynamic therapy to tumours also induce HO-1 expression in normal tissues.[22] Although some studies demonstrated that the incidence of tumours was associated with HO-1 promoter polymorphism,[23–27] little is known about the influence of the HO-1 expression level of normal tissues on the progressiveness of tumours. Therefore, our study was intended to determine the effect of HO-1 of normal tissues on tumour progressiveness by investigating the growth, metastasis and angiogenic potential of murine melanoma B16F10 cells in transgenic mice overexpressing HO-1 and its negative dominant mutant HO-1G143H, respectively.

Materials and methods

Experimental animals

The C57BL/6J mice were raised in sanitation cages and housed under natural day/night conditions (22°C, 55% humidity) and fed with a commercial pelleted diet *ad libitum*. Transgenic HO-1 mice overexpressing HO-1 protein were obtained by microinjecting gene fragment from pCAGGS-HO-1 plasmid between *SalI* and *DraI* sites. Likewise, transgenic HO-1G143H mice overexpressing HO-1 dominant mutant protein HO-1G143H were gained by microinjecting gene fragment from pCAGGS-HO-1G143H plasmid between *SalI* and *DraI* sites. The mice were passaged by mating between transgenic positive mice and wild-type C57BL/6J mice. The offspring was identified by PCR method and the negative mice were used as control. Eight-week-old mice were used for tumour inoculation experiments, and eight- and 18-week-old mice were used for leukocyte counts and flow cytometer experiments. All animal experiments were done in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and with the approval of the Scientific Investigation Board of Academy of Military Medical Sciences.

PCR identification and realtime-PCR analysis of transgenic mice

Mice were genotyped by PCR amplification using genome DNA obtained from mouse-tail biopsy samples. The transgene was detected with sense primer (5′-GGAGCGTCCACAGCCCGACA-3′), and antisense primer (5′- TGAGAGTGAGGACCCACTGGAGGAG-3′). Tail biopsies (~0.5 cm) were digested with 40 μg/mL of proteinase K in 500 μL of extraction buffer containing 100 mM Tris–HCl (pH 8.0), 200 mM NaCl, 5 mM EDTA and 0.2% SDS overnight at 55°C followed by phenol/chloroform extraction. After precipitation with isopropanol, DNA was washed with 70% ethanol and re-suspended in 50 μL of 10 mM Tris–HCl (pH 8.5). One microlitre ofDNA was subjected to PCR amplification (one cycle at 94°C for 5 min, 30 cycles at 94°C for 20 s, 60°C for 10 s and 72°C for 20 s, one cycle at 72°C for 10 min). The size of amplicon was 824 bp.

Realtime PCR was performed on an iQ5 Realtime PCR system (Bio-Rad, Hercules, CA, USA). Specific quantitative assays for HO-1 were carried out using SYBR Green dye. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene. The expected amplicon size was 62 bp and 177 bp, respectively. The sequences of all the primer pairs used[28] were as follows: HO-1 sense: 5′- CGCCTTCCTGCTCAACATT-3′ HO-1 antisense: 5′-TGTGTTCCTCTGTCAGCATCAC-3′; GAPDH sense: 5′-ATGACATCAAGAAGGTGGTG-3′ GAPDH antisense: 5′-CATACCAGGAAATGAGCTTG-3′. Complementary DNA (cDNA) was synthesized using 0.2 μg total RNA from each sample and reverse transcription reaction was carried out according to the instructions of ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). One microlitre of cDNA and gene-specific primers were added to SYBR Green PCR Master Mix (TOYOBO, Osaka, Japan), and subjected to PCR amplification[28] (one cycle at 50°C for 2 min, one cycle at 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min). The data were collected and analysed with iQ5 Software. Relative gene expression was determined using the delta–delta CT method.

Leukocyte counts and flow cytometer

The leukocyte counts were automatically operated by HEMAVET® HV950 Multispecies Hematology Systems (Drew Scientific Inc., UK). Blood samples were obtained from retro-orbital plexus of mice by venipuncture with a capillary pipette. About 200 μL blood from each mouse was collected in 0.5-mL tubes containing EDTA and misce bene.

Spleens were dissociated through a nylon mesh. Individual cell suspensions prepared from the spleen were re-suspended in PBS with 0.1% BSA and red cells were removed by lysis. Phenotypes of cells were determined for T lymphocytes – CD3-PerCP, CD4-FITC and CD8-PE. The CD45-APC leukocyte common antigen (LCA) staining was applied to distinguish the leukocytes from the residual red cells. Analysis was performed by FACS Calibre (Becton-Dickinson, Mountain View, CA, USA). Evaluation of data was performed using Flowjo software.

Tumour cell culture and inoculation

B16F10 murine melanoma cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum, glutamax (2 mmol/L), penicillin (100 U/mL) and streptomycin (0.1 μg/mL) at 37°C, in a humidified atmosphere with 5% CO_2.

All the procedures were performed according to the local bioethics commission protocols. For the first set, the mice were divided into three groups (control group: 10 male animals and nine female animals; transgenic HO-1 group: 14 male animals and 10 female animals; transgenic HO-1G143H group: 12 male animals and 11 female animals), and 100 μL of saline containing 2×10^5 B16F10 cells was

inoculated subcutaneously. Using a caliper, the tumour diameters were measured daily, and tumour diameter exceeding 20 mm was considered as the mouse endpoint.

In the second set, the mice were divided into three groups (control group: 8 male animals and 10 female animals; transgenic HO-1group: 11 male animals and 14 female animals; transgenic HO-1G143H group: nine male animals and 10 female animals), and injected with B16F10 cells into the tail vein (2×10^5 cells in 100 μL of saline). Animals were sacrificed on day 14 after inoculation, when the lungs were removed and fixed in Bouin solution for 24 h. Then the number of metastases was calculated by two researchers in a blinded manner.

Histochemistry

To show the expressions of HO-1 protein and HO-1G143H protein in transgenic mice, HO-1 of the spleens from transgenic mice and control mice was detected by immunohistochemical method. Frozen spleen tissues were cut at 4 μm, fixed in acetone at −20°C for 10 min and reacted with rabbit anti-HO-1 antibody for 1 h. Rhodamine-anti-rabbit IgG was used as second antibody. The expressions of HO-1 in spleens were detected by fluorescence microscopy.

To assess the influence of mice body overexpressing HO-1/HO-1G143H on pathological changes of tumours, three different sites were excised from subcutaneous tumour and metastatic lung nodules, respectively. The pathological changes were observed by a pathologist. The subcutaneous tumours and the lung tissues containing metastatic lung nodules were fixed in 4% formaldehyde solution for 24 h before being washed with PBS and sliced into multiple 1-mm sections. After dehydration through a graded ethanol series, slices were immersed in a 1:1 mixture of 100% ethanol and wax for 1 h at 37°C, followed by two 1-h incubations in pure wax at 37°C and then embedded in wax and cut into 3-μm-thick slices. Tissue sections were stained with hematoxylin and eosin for the assessment of pathological changes by light microscopy. To further access expressions of HO-1 on the lung with metastatic nodules, immunohistochemical stainings for lung sections were done according to our previous reported method.[29]

Statistical analysis

Results in groups were analysed using one-way analysis of variance (ANOVA) by SPSS 18.0 software, $P < 0.05$ values were considered significant.

Results

Production of transgenic mice lines and transgene mRNA and protein expression

Transgenic mice lines generated following the injection of target fragments containing HO-1 and HO-1G143H genes were named TgHO-1 mice and TgHO-1G143H mice, respectively. The offspring of transgenic mice inbred with wild mice was identified by PCR using primers specific to HO-1 (Figure 1a). The expression of the transgenes was assessed using an RT-realtime-PCR assay on total RNA extracted from lung tissues. As Figure 1b shows, the mRNA expression of HO-1 in TgHO-1 mice was 10 times that of control mice, and the mRNA expression of HO-1 in TgHO-1G143H mice increased 60-fold compared with that of control mice. According to the fluorescence intensity (Figure 4a), we can see that HO-1 protein and HO-1G143H protein in transgenic mice are also overexpressed, respectively, compared with control mice. Furthermore,

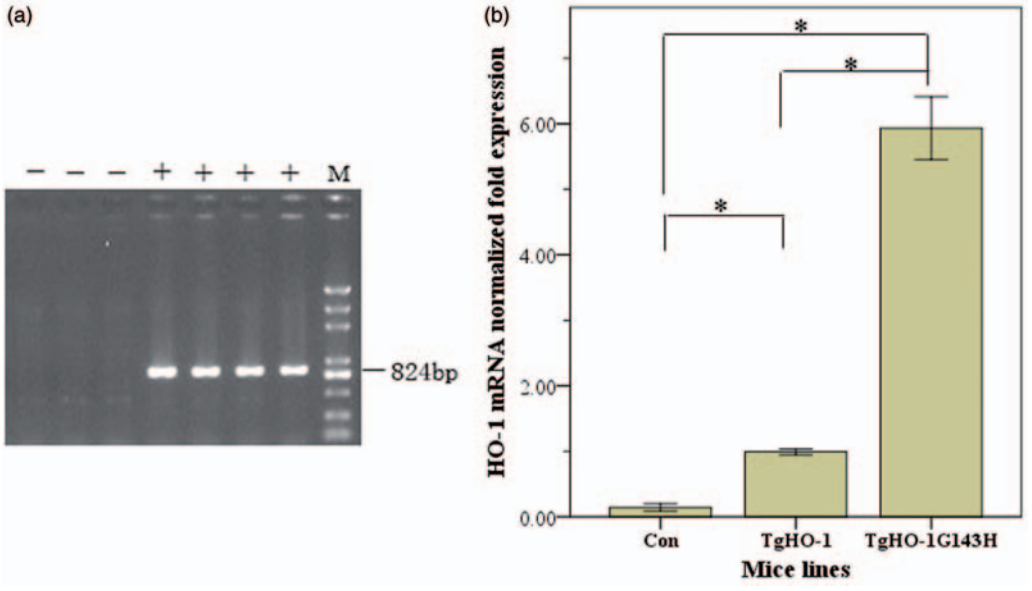

Figure 1 Screening of transgenic mice and detection of HO-1 mRNA. (a) The genomic DNA extracted from a brood offspring was identified. The PCR products were analysed on an agarose gel stained with ethidium bromide. −: negative mice; +: positive transgenic mice; M: DL2000 DNA ladder. (b) HO-1 mRNA expression of transgenic mice. The quantitives of mice HO-1 mRNA of transgenic mice were normalized as described under 'Methods' section. Error bars represent standard error of mean of six mice in each group. Con: control mice; TgHO-1: transgenic HO-1 mice line; TgHO-1: transgenic HO-1G143H mice line; *means significant differences ($P < 0.05$). (A color version of this figure is available in the online journal)

expression intensity in TgHO-1G143H mice is higher than that in TgHO-1 mice.

The phenotypes of transgenic mice overexpressing HO-1 and HO-1G143H

There was no significant difference in phenotypes of transgenic mice before 24 weeks, compared with wild-type mice.[30] The TgHO-1G143H mice were smaller than wild-type littermates only after 24 weeks. However, this transgenic mice arose splenomegalia and iron deposition in the liver and kidney at 12-month-old. Since HO-1 is regarded as an anti-inflammatory enzyme, we decided to investigate the counts of white blood cells in peripheral blood and splenic CD4+:CD8+ T-cell ratios of mice overexpressing HO-1 and HO-1G143H at eight and 18 weeks old, respectively.

There was no difference in the counts of white blood cells in peripheral blood and splenic CD4+:CD8+ T-cell ratios of eight-week-old mice (data not shown). For 18-week-old mice, the white blood cell counts of peripheral blood of TgHO-1 mice were somewhat decreased compared with the control mice and significantly lower than those of TgHO-1G143H mice. Furthermore, the TgHO-1G143H mice exhibited a significant increase of white blood cell counts compared with control mice (Figure 2a). The outcome of flow cytometer analysis for T lymphocytes (Figure 2b) showed the splenic CD4+:CD8+ T-cell ratios of TgHO-1G143H mice were significantly increased compared with TgHO-1 mice and control mice. Meanwhile, the splenic CD4+:CD8+ T-cell ratios of TgHO-1 mice were also somewhat decreased compared with the control mice.

Thus, eight-week-old mice used for tumour inoculation experiments in this study just excluded the possibility that overexpression of HO-1 or HO-1G143H themselves could affect life span and therefore influence survival in the tumour-bearing mice.

Overexpressions of HO-1/HO-1G143H of mice had no significant effect on the survival rate of tumour-bearing mice except for pathological changes of subcutaneous tumour

To investigate the effect of HO-1 of normal tissues on tumour growth, B16F10 melanoma cells were injected subcutaneously into the back of syngeneic C57BL/6J mice (2×10^5 cells per animal) overexpressing HO-1 and HO-1G143H, respectively. Tumour emergence time and tumour sizes were recorded, and the time when tumour diameter exceeded 20 mm was considered the death time of mice. There was no significant difference between the tumour emergence time of control mice (12.94 ± 2.91 days), TgHO-1 mice (13.75 ± 5.54 days) and TgHO-1G143H mice (14.35 ± 4.35 days) or between the survival rate of control mice, TgHO-1 mice and TgHO-1G143H mice (Figure 3). Median survival of animals was 22 days, 26 days and 25 days after tumour cell inoculation for control mice, TgHO-1 mice and TgHO-1G143H mice, respectively (Kaplan–Meier Wilcoxon analysis). These data demonstrated that overexpression of neither HO-1 nor HO-1G143H in mice significantly changed the tumour emergence time and the survival time of tumour-bearing mice.

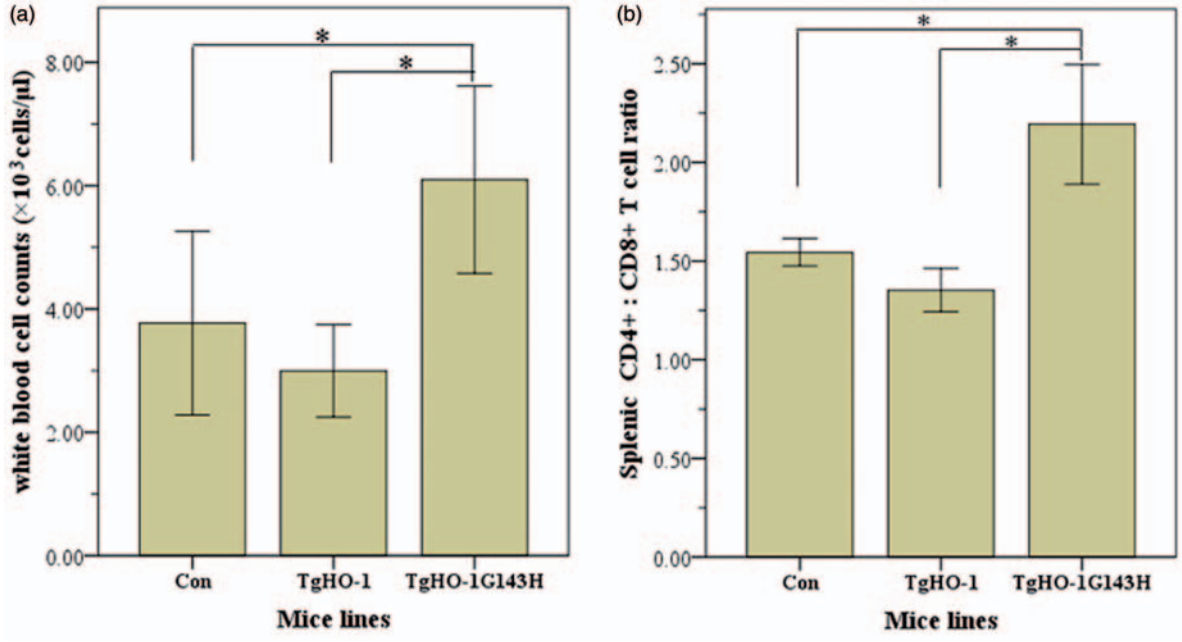

Figure 2 Markers of inflammation in wild-type mice, TgHO-1 mice and TgHO-1G143H mice. (a) The average of white blood cell counts (WBC). (b) Splenic CD4+:CD8+ T-cell ratios analysis. Each group contained seven to eight mice (approximately 18 weeks of age). Error bars represent standard error of each group. Con: control mice; TgHO-1: transgenic HO-1 mice line; TgHO-1: transgenic HO-1G143H mice line; *means significant differences ($P < 0.05$) compared with TgHO-1G143H mice. (A color version of this figure is available in the online journal)

The subcutaneous tumour from control mice showed more inflammatory cells and fibrocyte infiltration, and the tumour from TgHO-1G143H mice showed far more inflammatory cells and fibrocyte infiltration and more small vessels (Figure 4b). However, the tumour from the transgenic HO-1 mice developed limited inflammatory cell infiltration, but more bulky vessels could be seen (Figure 4b). These data demonstrated that HO-1 also played an anti-inflammatory role in white cell infiltration and pro-angiogenic action in subcutaneous tumour.

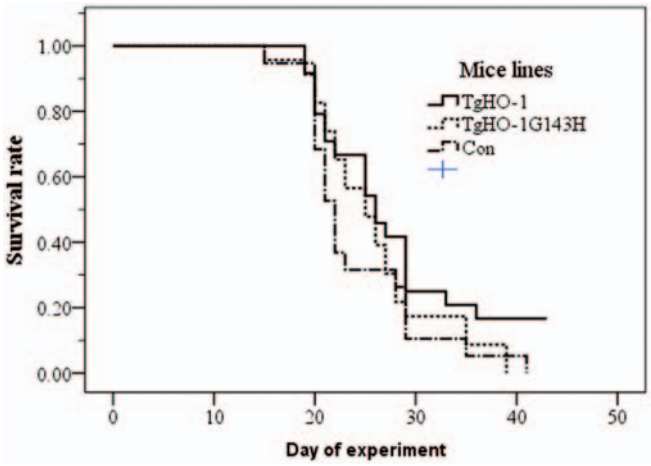

Figure 3 Survival curves of mice injected subcutaneously with B16F10 melanoma cells (2×10^5 cells/per animal). The mice were divided into control mice group, TgHO-1 mice group and TgHO-1G143H mice group
Con: control mice; TgHO-1: transgenic HO-1 mice line; TgHO-1: transgenic HO-1G143H mice line; There was no significant difference between the three groups ($P > 0.05$)

Overexpressions of HO-1/HO-1G143H of mice interfered with tumour lung metastasis and their pathological changes

To assess the effect of HO-1 of a mouse body on the ability of tumour to home and grow in distant organs, we injected B16F10 cell lines into the tail vein of mice overexpressing HO-1 and HO-1G143H (2×10^5 cells/per animal), respectively. Such treatment resulted in different formation of tumours within the lungs in the three groups. We compared the number of metastases at day 14 after inoculation. As shown in Figure 5, the number of lung metastatic nodules strongly increased in TgHO-1G143H mice compared with TgHO-1 mice and control mice. Although there was no significant difference in the number of lung metastatic nodules between TgHO-1 mice and control mice, the number of lung metastatic nodules of TgHO-1 mice was remarkably smaller than that of control mice, suggesting that overexpression of HO-1 in mice could inhibit melanoma lung metastasis, and that overexpression of HO-1G143H in mice could promote melanoma lung metastasis, which contributed to the shortened survival time of mice bearing-metastatic tumours.

In this study, the pathological changes of metastatic lung nodules in mice were assessed. These nodules grew surrounded with central thick vessels, and there was no abnormality in the lungs of control mice (Figure 6, control mice). However, there was apparent focal lymphocytosis and slight congestion in the lungs of TgHO-1 mice (Figure 6, TgHO-1 mice). The pathological changes of TgHO-1G143H mice were much more significant than those of TgHO-1 mice and control mice (Figure 6, TgHO-1G143H mice). For example, tumour nodes grew besides thick vessels; the tumour cells displayed a bigger nucleus/plasma ratio, chromatin unevenness and obvious nucleus dividing phase and heteromorphism; spindle cells, lymphoid cells and vacuole cells existed; congestion was in vessels of the

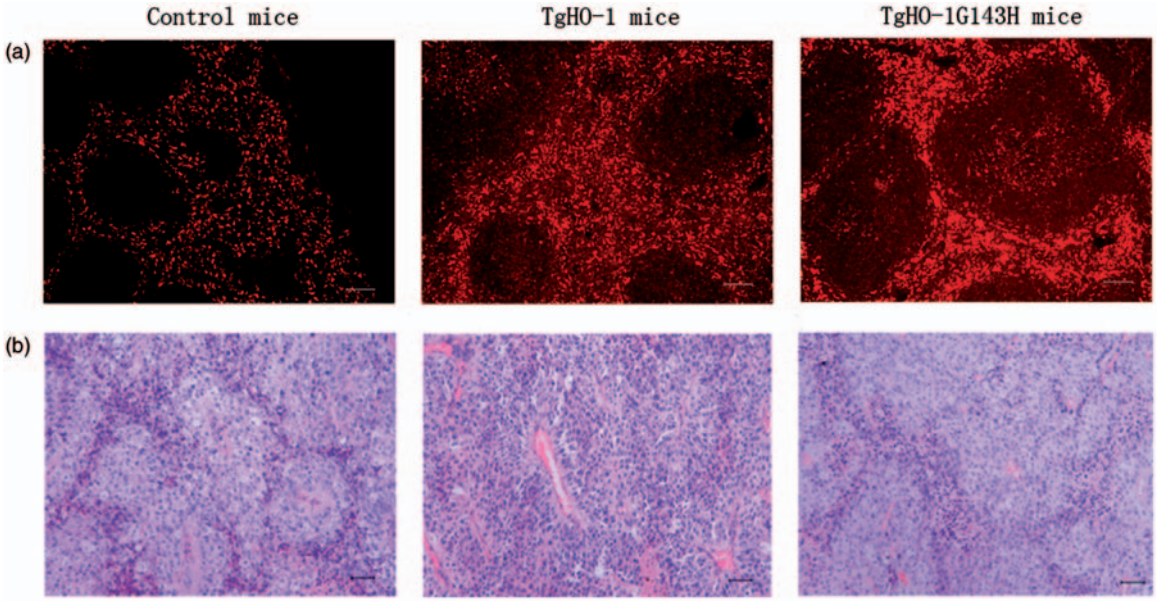

Figure 4 (a) Representative immunofluorescence of frozen sections showing the expression level of HO-1 in the spleens from control mice, TgHO-1 mice and TgHO-1G143H mice (×200). (b) Representative H&E wax specimens showing the pathological changes of subcutaneous tumours from control mice, TgHO-1 mice and TgHO-1G143H mice (×200). Bar represents 100 μm. (A color version of this figure is available in the online journal)

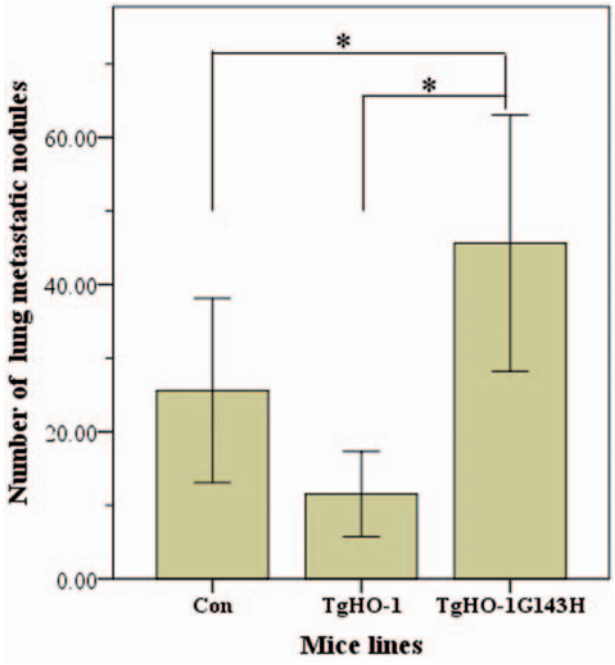

Figure 5 Number of metastatic lung nodules in mice inoculated intravenously with B16F10 melanoma cells (2×10^5 cells/per animal). The mice were divided into control mice group, TgHO-1 mice group and TgHO-1G143H mice group. Error bars represent standard error of mean of each group. (A color version of this figure is available in the online journal)
Con: control mice; TgHO-1: transgenic HO-1 mice line; TgHO-1: transgenic HO-1G143H mice line; *means significant differences ($P < 0.05$) compared with TgHO-1G143H mice

lung stroma; pulmonary alveoli were in compensatory extension. These data suggested HO-1 could play a beneficial role in pathological changes of metastatic lung nodules.

Discussion

HO-1 is an inducible enzyme, the expression and function of which in tumours are often a chief concern. However, the function of HO-1 expression in normal tissues on tumours is less reported. It was showed that the incidence of oral squamous cell carcinoma in betel chewers and lung adenocarcinoma in cigarette smokers was lower in a population with a shorter sequence of (GT)n in the HO-1 promoter.[23,24] It seems that this is due to the protection of HO-1 on healthy cells. Furthermore, chemotherapy, radiotherapy and photodynamic therapy to tumours also induce HO-1 expression in normal tissues.[22] Unfortunately, the influence of the HO-1 expression level of normal tissues on progressiveness of tumours remains a mystery. Our results demonstrated for the first time that HO-1 affected tumour progressiveness by inoculating melanoma B16F10 cells into the TgHO-1 mice overexpressing HO-1 and TgHO-1G143H mice overexpressing HO-1G143H, respectively.

The mutant protein HO-1G143H could bind to heme rather than transfer electrons necessary to execute the catalytic reaction of HO-1,[31] displaying a phenotype to dominant-negative effects. In our previous papers, the effects of HO-1G143H in cell lines[30,32] and transgenic mice[30] have been detected. HO activities in QT6 cells transfected with pCAGG-HO-1-G143H could not be detected at all.[30] Furthermore, it decreased in BGC-823cells compared with control cells, and the possible mechanisms of it have been discussed in this section[32]. Since the amount of mRNA and protein of HO-1 in TgHO-1G143H mice were overexpressed in TgHO-1G143H mice, the wild-type HO-1 could not compete to bind heme with HO-1G143H protein. Consequently, there were few anti-inflammatory products of HO-1 in TgHO-1G143H mice.

There was no significant difference in the phenotypes of eight-week-old transgenic mice used for tumour inoculation experiments in present study. In order to confirm anti-inflammatory role of HO-1, we further detected the counts of white blood cells in peripheral blood and splenic CD4+:CD8+ T-cell ratios of 18-week-old mice. For 18-week-old mice, the white blood cell counts and splenic CD4+: CD8+ T-cell ratios of TgHO-1G143H mice were significantly increased compared with wild-type mice that displayed the same inflammatory phenotype of HO-$1^{-/-}$ mice.[33–35] On the other hand, the overexpression of HO-1 mRNA in TgHO-1 mice resulted in a slightly smaller number of WBC and splenic CD4+:CD8+ T-cell ratios compared with the wild-type mice, as in the clonal deletion of CD4+ T cells of HO-1 function.[36] The exact mechanisms underlying the anti-inflammatory functions of the HO-1 have not been fully elucidated. However, the signalling action of CO[37–40] combined with the antioxidant properties of biliverdin/bilirubin[41,42] and the sequestration of iron by ferritin[43] all contributed to suppression of inflammation. Phenotypes of these transgenic mice demonstrated again that the anti-inflammatory characteristics of HO-1 might be mainly displayed by the products of HO-1 catalysates.

Through the production of an array of growth factors, proteases and angiogenic mediators, leukocytes in the tumour microenvironment promote tumour growth, angiogenesis and metastasis,[44] and leukocyte infiltration within the tumour is a negative prognostic factor for the patients.[45] For example, besides being able to destroy tumour cells,[46] macrophages can promote growth of tumours by producing angiogenic factors such as IL-1β, IL-8, bFGF, VEGF or EGF,[47] and by releasing proteolytic enzymes, which degrade extracellular matrix and stimulate tumour cell migration. *In vitro* studies have shown both reduced pro-inflammatory cytokine (TNF-α, IL-1β, MIP-1β and IL-6) release and increased IL-10 expression in LPS stimulated in macrophages following HO-1 overexpression and/or CO exposure.[38,39,48] Thus, it was presumed that HO-1 might be potentially beneficial for tumour patients. In this study, there was less inflammatory cell infiltration in tumours of TgHO-1 mice but much more inflammatory cell infiltration in tumours of TgHO-1G143H mice. These results seemed to conflict with the finding that there was less-pronounced leukocyte infiltration in tumours overexpressing HO-1.[13] We speculated that the benefit of normal tissues of HO-1 to tumour leukocyte infiltration resulted from CO which can diffuse through cell membranes, and the biliverdin/bilirubin which could be transported by blood flow.

Apart from resisting leukocyte infiltration, HO-1 is recognized as a pro-angiogenic enzyme. In this study, it was discovered that there were bulky vessels in

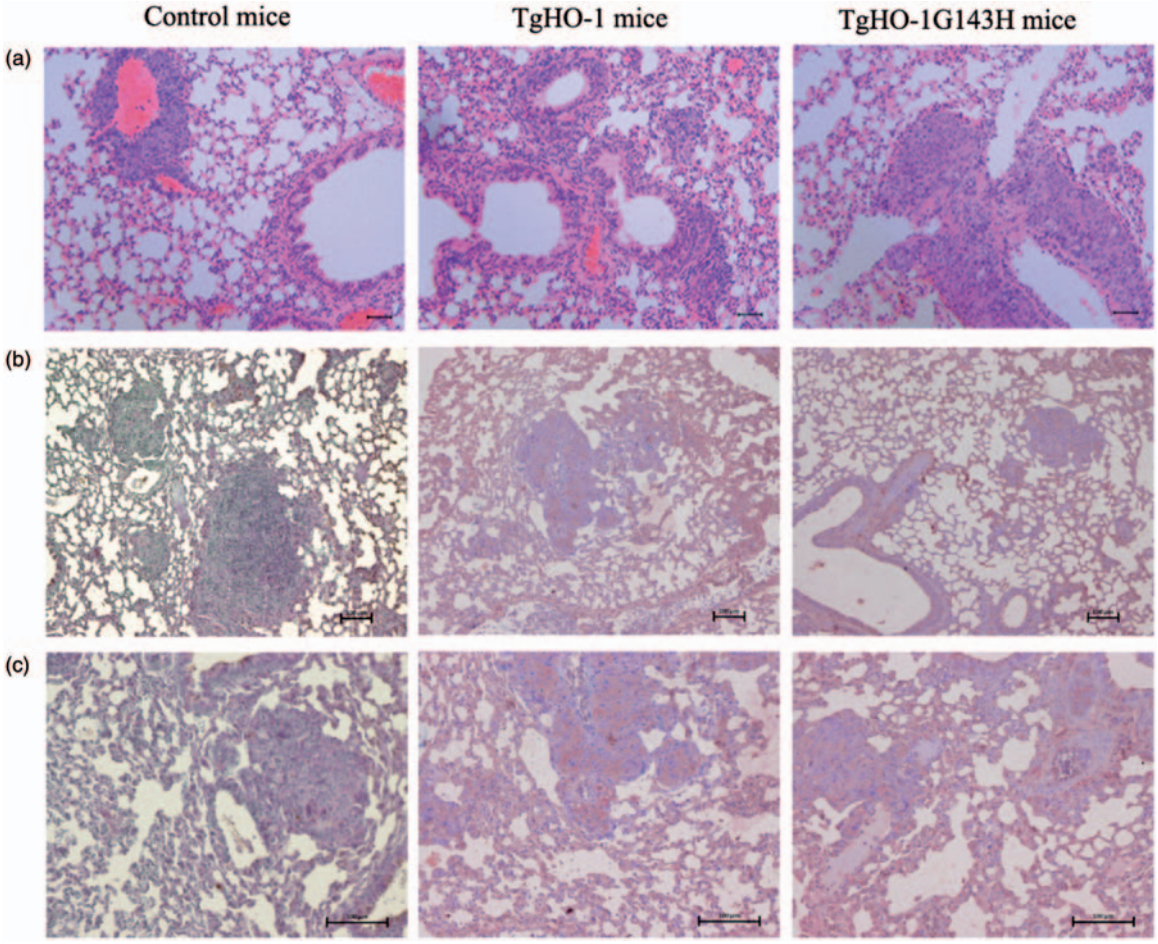

Figure 6 (a) Representative H&E wax specimens showing the pathological changes of tumour lung metastases from control mice, TgHO-1 mice and TgHO-1G143H mice (×200). (b) Representative immunohistochemistry of wax specimens showing the expression level of HO-1 in the spleens from control mice, TgHO-1 mice and TgHO-1G143H mice (×100). (c) Representative immunohistochemistry of wax specimens showing the expression level of HO-1 in the spleens from control mice, TgHO-1 mice and TgHO-1G143H mice (×200). Bar represents 100 μm. (A color version of this figure is available in the online journal)

subcutaneous tumours and tumour nodes in TgHO-1 mice and much more small vessels, which was consistent with the report that the subcutaneous melanoma overexpressing HO-1 had better vascularization.[13] These results further demonstrated that the pro-angiogenic action of HO-1 was mainly mediated by CO.[49,50] However, angiogenesis plays a crucial role during the growth and spreading of tumours. This pro-angiogenic action of HO-1 would be deleterious to tumour-bearing patients.

Since melanoma overexpressing HO-1 shortened the survival of the tumour-bearing mice,[13] there was reason to speculate that HO-1 overexpression in animals may help defend against tumours. However, there was no significant difference in tumour emergence time and survival rates of mice between wild-type mice, TgHO-1 mice and TgHO-1G143H mice inoculated subcutaneously with B16F10 cells. Nevertheless, it is worth noting that TgHO-1 mice survived longer (median survival of time, 26 days) than control mice (median survival of time, 22 days). Furthermore, overexpressing HO-1 in TgHO-1 mice obviously inhibited tumour growth, compared with control mice and TgHO-1G143H mice (data not shown). These results offered more evidence on the protective role of HO-1. However, it remains unknown why TgHO-1G143H mice unexpectedly survived longer (median survival of time, 25 days) than control mice. The results of melanoma lung metastases in transgenic mice indeed demonstrated that HO-1 overexpression in normal tissues could contribute to the protection against tumour metastases. The TgHO-1 mice intravenous inoculation of B16F10 cells developed fewer and clearly smaller nodules than their counterparts. Conversely, increased numbers and sizes of lung metastases emerged in TgHO-1G143H mice, suggesting that the inhibitory action played by HO-1 on tumour metastases also depended on CO and biliverdin/bilirubin.

Conclusion

We showed that the overexpression of HO-1 potently inhibited melanoma cancer aggressiveness by inhibiting metastases and inflammatory cell infiltration in TgHO-1 mice, and that overexpression of HO-1G143H resulted in inflammatory phenotype and promoted tumour metastasis. These beneficial roles of HO-1 might be mainly attributed to HO-1 catalysates. However, increased activity of HO-1 in animals could not significantly increase the survival rate of

tumour-bearing mice. Therefore, we supposed that upregulation of HO-1 in normal tissues might be beneficial for patients bearing melanoma metastasis.

Author contributions: QL and HZ conceived and designed the experiments, QL, BW, YY, GC, XW and PW performed the experiments, QL, analysed the data, QL, BW, YY and XG contributed reagents/materials/analysis tools, QL wrote the paper and QL and BW contributed equally to this work.

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