Myeloid heme oxygenase-1 promotes metastatic tumor colonization in mice

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Heme oxygenase-1 (HO-1) is a heme degradation enzyme with antioxidant and immune-modulatory functions. HO-1 promotes tumorigenesis by enhancing tumor cell proliferation and invasion. Whether HO-1 has an effect on cancer progression through stromal compartments is less clear. Here we show that the growth of tumor engrafted subcutaneously in syngeneic mice was not affected by host HO-1 expression. However, lung metastasis arisen from subcutaneous tumor or circulating tumor cells was significantly reduced in HO-1+/− mice comparing to wild type (WT) mice. The reduced lung metastasis was also observed in B6 mice bearing HO-1+/− bone marrow as comparing to WT chimeras, indicating that HO-1 expression in hematopoietic cells impacts tumor colonization at the metastatic site. Further experiments demonstrated that the numbers of myeloid cells recruited to pulmonary premetastatic niches and metastatic loci were significantly lower in HO-1+/− mice than in WT mice. Likewise, the extents of tumor cell extravasation and colonization at the metastatic loci in the early phase of metastasis were significantly lower in HO-1+/− mice. Mechanistic studies revealed that HO-1 impacted chemotactant-induced myeloid cell migration by modulating p38 kinase signaling. Moreover, myeloid HO-1-induced expressions of vascular endothelial growth factor and interleukin-10 induced metastasis of tumor cell transendothelial migration and STAT3 activation in vitro. These data support a pathological role of myeloid HO-1 in metastasis and suggest a possibility of targeting myeloid HO-1 for cancer treatment.

Myeloid cells have been shown to influence cancer initiation and progression in many ways. The tumor-associated macrophages produce angiogenic and growth factors and the matrix metalloproteases to promote tumor cell growth, angiogenesis and metastasis. These cells also induce immune suppression by producing immunosuppressive cytokines, such as interleukin-10 (IL-10) and transforming growth factor-beta. Moreover, the accumulation of myeloid-derived suppressor cells (MDSC) in the tumor microenvironment exerts a profound effect on tumor progression through their potent immunosuppressive activity on T cells. In addition to their multiple roles in primary tumors, increasing evidence has revealed the involvement of myeloid cells in supporting seeding, engulfment and survival of metastatic tumor cells in distant sites. Studies have shown that soluble factors secreted by the primary tumor can prime distant organs by inducing the expressions of fibronectin and chemoattractants in focal areas, which subsequently promotes the recruitment of myeloid cells to form a receptive pre-metastatic niche for disseminating tumor cell invasion and growth. Moreover, a population of inflammatory monocytes/macrophages can be recruited to the metastatic sites early in the metastasis process to facilitate the seeding and survival of tumor cells. These findings highlight the pivotal role of myeloid cells in cancer metastasis. Increasing our understanding of the intrinsic factor(s) that modulate the prometastatic function of myeloid cells could pave the way for new therapeutics for metastatic disease.
performed additional experiments to disclose the molecular and cellular mechanisms involved.

Materials and Methods

**Tumor cell culture.** Lewis lung carcinoma (LLC) and B16F10 melanoma cells were cultured in DMEM supplemented with 10% FBS. GFP-expressing LLC cells were established by infection with lentivirus bearing the GFP gene and selected with 4 μg/mL puromycin. To label B16F10 cells with fluorescent dyes, cells were incubated with 10 μM CellTracker Orange CMOTMR (5-(and-6)-((4-Chloromethyl)Benzoyl)Amino)Tetramethylrhodamine) (Invitrogen, Carlsbad, CA, USA) or CellTracker Green CMFDA (5-Chloromethylfluorescein Diacetate) (Invitrogen) in serum-free DMEM at 37°C for 1 h, followed by incubation in complete medium for another 30 min. After 3 PBS washes, cells were trypsinized and used for subsequent experiments immediately.

**Bone marrow-derived macrophages.** Bone marrow (BM) cells were removed from the femurs and tibias of mice and the induction of macrophage differentiation was performed as described.(22)

**Preparation of conditional medium.** Lewis lung carcinoma and B16F10 cells (3 × 10⁵) plated in a 10-cm petri dish or 5 × 10⁵ bone marrow-derived macrophages (BMDM) plated in a 3.5-cm dish were cultured in serum-free DMEM (phenol red free) containing 1% BSA for 18 h. Medium were collected, passed through a 0.22-μm filter, and stored as aliquots at −80°C prior to use.

**Animal experiments.** The animal experimental procedures were approved by the Institutional Animal Care and Utilization Committee of the Academia Sinica, Taiwan. WT and HO-1+/− mice, originally on B6/129Sv mixed background, were backcrossed 10 generations onto the C57BL/6J genetic background. To generate B6 chimeric mice reconstituted with WT or HO-1+/− BM, we performed BM transplantation with lethally irradiated C57BL/6J mice (6-week-old male) as described previously.(23) To examine the spontaneous lung metastasis, we injected 2 × 10⁵ LLC cells subcutaneously into the right flanks of mice (8–10-week-old male) for 3 weeks. The subcutaneous primary tumors and lungs were removed for further assessments. To perform the experimental lung metastasis, GFP-labeled LLC cells (1 × 10⁵) or B16F10 cells (2 × 10⁵) were injected i.v. via tail veins of animals. Mouse were sacrificed at indicated times and lung tissues were examined. To assess premetastatic niche formation, mice received i.p. injection of control medium or tumor cell-conditioned medium (CM) (300 μL/mouse) for 10 consecutive days prior to sacrifice. In some experiments, mice received an i.v. injection of fluorescent dye-labeled B16F10 cells for indicated times. The lung tumor metastases were examined using a fluorescent stereo microscope (STEREO Lumar V12; Carl Zeiss, Munich, Germany), and quantified by MetaMorph software, Molecular Devices, Sunnyvale, CA, USA.

**Real time quantitative PCR.** RNA isolation, reverse transcription reaction and real-time PCR were performed as described previously.(23) The primer pairs used are summarized in Table S1.

**Transwell migration assay.** Mouse splenic CD11b⁺ cells were isolated using the EasySep mouse CD11b positive selection kit (Stemcell Tech, Vancouver, BC, Canada). Cell migration was assessed using 24-well Transwell plates as described previously.(24)

**Immunohistochemistry and immunofluorescence staining.** Paraffin-embedded sections were blocked with 5% normal goat serum, followed by incubation with rat anti-mouse CD11b (eBioscience San Diego, CA, USA) for 1 h at 37°C. After 3 PBS washes, the antigen-antibody complex was detected by incubation with horseradish peroxidase-conjugated anti-rat IgG and stained with diaminobenzidine. For immunofluorescence staining, lung cryosections were blocked with 5% normal goat serum, followed by incubation with rat anti-mouse CD11b (eBioscience) or rabbit anti-mouse F4/80 antibody at 37°C for 1 h. After 3 PBS washes, sections were incubated with Alexa Fluor 555-conjugated goat anti-rat (Cell Signaling Technology, Danvers, MA, USA) or Alexa Fluor 488-conjugated goat anti-rabbit (Molecular Probes, Saint Aubin, France) antibody for another 1 h at room temperature in the dark. Nuclei were then stained with DAPI and sections examined by fluorescence microscope (BX51; Olympus, Tokyo, Japan) at 200× magnification.

**Confocal microscopy.** Lung cryosections blocked with 5% goat serum were incubated with rat anti-CD11b and rabbit anti-phospho-STAT3 (Cell Signaling) at 37°C for 1 h. After 3 PBS washes, sections were incubated with Alexa Fluor 647-conjugated goat anti-rat (Cell Signaling) and Alexa Fluor 488-conjugated goat anti-rabbit antibodies (Molecular Probes, Saint Aubin, France) for another 1 h at room temperature in the dark. Nuclei were stained with DAPI and examined by confocal microscope (LSM 510 META; Carl Zeiss, Jena, Germany) at 10× magnification.

**In vivo tumor cell extravasation.** 1 × 10⁶ CMFDA-labeled B16F10 cells were injected i.v. into mice for 4 h. Lungs were fixed by perfusion with PBS containing 4% paraformaldehyde and 0.3% Triton X-100 for 15 min and then washed by PBS containing 0.1% Triton X-100. Lungs cut in small pieces were incubated with Isolecitin IB4 Alexa Fluor 647 conjugates (1:50; Invitrogen) at 4°C for 24 h. After 3 PBS washes, tissues were treated with FocusClear (CellExplorer, Hsinchu, Taiwan) for an additional 24 h prior to scanning with a confocal microscope (Ultra View ERS-3FE). Images from at least eight different fields per lung were taken, and the 3-D reconstruction was performed using Velocity (Perkin Elmer, Waltham, MA, USA).

**Tumor transendothelial migration assay.** The transwell with pore size of 8 μm (Corning, Union city, CA, USA) was coated with 125 μg/mL Matrigel, followed by seeding with 5 × 10⁴ SV40 transformed mouse lymph node endothelial cells (SVEC). After 42 h in culture, 1 × 10⁵ CMFDA-labeled B16F10 cells were added onto the SVEC monolayer of the upper chamber. The lower chamber was filled with WT and HO-1−/−BMDM-CM alone or containing indicated amounts of goat control IgG or goat anti-VEGF antibody as indicated. After 8 h of incubation at 37°C, B16F10 cells migrated to the lower surface of the transwell membrane were fixed with 4% paraformaldehyde and examined by fluorescence microscopy. The photos from five different fields (× 100 magnification) of each transwell were taken and cells counted and data presented as cell number per field.

**Western blot analysis.** Western blot analysis was performed as described previously.(23) The primary antibodies used were: anti-phospho-p38, anti-p38, anti-phospho-STAT3 and anti-STAT3 (all from Cell Signaling).

**Statistical analysis.** Results were expressed as the means ± SE. Differences between groups were examined for statistical significance using Student’s t-test or ANOVA. A P-value <0.05 was considered statistically significant.

Results

Hematopoietic heme oxygenase-1 promotes lung metastasis. When the syngeneic WT and HO-1−/− mice received
subcutaneous injection of LLC cells in the dorsal right flanks for 3 weeks, the subcutaneous tumors grown in WT and HO-1+/−/C0 mice were not significantly different (Fig. S1a,b). However, examination of the lung sections revealed more metastases developed in WT mice than HO-1+/−/C0 counterparts (Fig. S1c,d). We then performed the experimental lung metastasis assay by directly injecting GFP-LLC cells through tail veins of mice. As shown in Figure 1(a,b), lung metastasis at 2 weeks after LLC inoculation was significantly greater in WT mice than HO-1+/−/C0 mice. Similar results were obtained when an experiment was performed with B16F10 cells (Fig. 1c,d).

To further examine whether hematopoietic HO-1 expression impacts lung metastasis assay by directly injecting GFP-LLC cells through tail veins of mice. As shown in Figure 1(e,f), the lung metastases developed at 10 days post-B16F10 inoculation were significantly lower in HO-1+/−/C0 chimeras compared to WT chimeras, supporting the involvement of hematopoietic HO-1 in facilitating tumor colonization at metastatic sites.

Myeloid heme oxygenase-1 influences premetastatic niches formation. To examine whether host HO-1 has an influence on the establishment of a premetastatic niche in distant organ induced by tumor-derived soluble factors (5–8) we subjected WT and HO-1+/−/mice to i.p. injection of CM collected from tumor cells for 10 consecutive days as reported previously. (5) The accumulation of CD11b+ myeloid cells in the lung parenchyma was then examined by immunofluorescence staining. As illustrated in Figure 2(a,b), both LLC- and B16F10-CM induced substantial myeloid cell infiltration in the lungs, which was more prominent in WT mice. Flow cytometric analysis of these recruited cells demonstrated that a significant cell population expressed both CD11b and Gr-1 (Fig. S2a,b), which are the characteristic markers of MDSC. To investigate whether the accumulation of myeloid cells augments the lung metastasis of tumor cells, both WT and HO-1+/−/mice were pretreated with B16F10-CM for 10 days, followed by i.v. injection of B16F10 cells. Results showed that lung metastasis was significantly facilitated with more tumor nodules found in the WT group (Fig. 2c,d). Immunohistochemistry again revealed more CD11b+ myeloid cells clustered around B16F10 cells in the lungs of WT mice (Fig. 2e).

Heme oxygenase-1 influences tumor cell extravasation and STAT3 activation in metastatic sites. Myeloid cells, particularly inflammatory monocytes/macrophages, have been shown to promote tumor cell extravasation and survival during the early phase of metastasis. (9,10) To assess the potential impact of myeloid HO-1 on these processes, we first examined the mobilization of monocytes/macrophages to lungs of WT and HO-1+/−/mice after i.v. injection of CMTMR-labeled B16F10 cells. As demonstrated in Figure 3(a,b), equivalent numbers of tumor cells were found in the lungs of WT and HO-1+/−/mice at 4 h after B16F10 cell inoculation. After 24 h, tumor cells remaining in the lungs were significantly reduced. Nevertheless, more tumor cells were detected in the lungs of WT mice.
compared to HO-1+/− mice. An immunostaining experiment revealed the recruitment of significant numbers of F4/80+ macrophages to lungs of mice at 4 h post-B16F10 cell injection (Fig. 3a,c). Notably, the macrophage infiltration was more prominent in WT mice. Although macrophages declined at a later time point (24 h), they remained higher in the WT mice than in their HO-1+/− counterparts. To examine whether tumor cell extravasation is correlated with macrophage infiltration, the lungs of mice subjected to infusion of CMFDA-labeled B16F10 cells for 4 h were incubated with red fluorescent dye-conjugated isolectin B4 to stain the vasculature, followed by examination with 3-D confocal microscopy. It was shown that significant percentages of B16F10 cells were present within the blood vessels in both groups of mice (Fig. 4a,b). Nevertheless, the number of tumor cells located in the extravascular areas was much higher in WT mice compared to these in HO-1+/− mice (14.56 ± 1.26% vs 10.01 ± 1.51%, P = 0.019) at this early time point.

STAT3 signaling activated by many growth factors and cytokines is crucial for cancer cell survival and proliferation.25,26 To assess whether macrophages recruited to the metastatic foci can promote tumor cell survival by activating STAT3 signaling, we performed confocal immunofluorescence with lung sections of mice receiving B16F10 cell infusion for 48 h. As illustrated in Figure 4(c), the positive immunostain for phospho-STAT3 was most prominent in tumor cells arrested in the lungs of WT mice compared to these in HO-1+/− mice. Moreover, the percentage of tumor cells with positive stain of phospho-STAT3 was much higher in WT mice (Fig. 4d).

Heme oxygenase-1 affects migration response of myeloid cells. To understand the molecular basis of how more myeloid cells were recruited to lungs of WT mice following tumor-CM treatment, we first examined the expressions of pulmonary genes involved in the recruitment of myeloid cells. Consistent with previous reports,5–7 the pulmonary expressions of fibronectin, lysyl oxidase and chemoattractants, S100A8 and S100A9, were significantly increased in mice treated with tumor-CM (Fig. S3). However, the extents of induction were comparable between WT and HO-1+/− mice. We then examined whether HO-1 influences the migration responses of myeloid cells toward chemoattractants, particularly S100A8/S100A9, which are the major chemoattractants mediating myeloid recruitment via p38 kinase signaling.60 As shown in Figure S4(a), HO-1 protein expression in splenic CD11b+ cells isolated from WT mice was much higher than that of CD11b+ cells from HO-1+/− mice. Likewise, WT-CD11b+ cells exhibited greater...
Fig. 3. Heme oxygenase-1 (HO-1) influences monocyte/macrophage mobilization to metastatic site. Wild type (WT) and HO-1\textsuperscript{+/−} mice (\(n=5\) /group) received an i.v. injection of PBS (control) or \(1 \times 10^{6}\) CMTMR-labeled B16F10 cells. After 4 and 24 h, lungs were harvested and tissue sections were subjected to immunofluorescence staining with F4/80 antibody. (a) Representative images showing B16F10 cells (red) and F4/80\textsuperscript{+} macrophages (green) in the lungs of treated mice. Bar = 100 \(\mu m\). (b) Quantitative results of B16F10 cells in various groups. *\(P < 0.01\) versus WT group. (c) Quantitative results of F4/80\textsuperscript{+} macrophages in various groups. *\(P < 0.01\) versus control group of same genotype; †\(P < 0.03\) versus WT group.

Fig. 4. Heme oxygenase-1 (HO-1) influences extravasation and STAT3 activation of metastatic tumor cells. (a,b) Wild type (WT) and HO-1\textsuperscript{+/−} mice (\(n=5\) /group) received an i.v. injection of \(1 \times 10^{6}\) CMFDA-labeled B16F10 cells for 4 h. Pulmonary vasculature was labeled by isolectin IB4 Alexa Fluor 647 conjugates. (a) Representative 3-D confocal images of lungs. Bar = 12 \(\mu m\). (b) The percentages of intravascular and extravascular tumor cells were determined. *\(P < 0.02\) versus WT group. (c) WT and HO-1\textsuperscript{−/−} mice received an i.v. injection of \(1 \times 10^{6}\) CMTMR-labeled B16F10 cells for 48 h. The lung sections were subjected to immunofluorescence staining with antibodies against p-STAT3 and CD11b, respectively. Data shown is the representative image. (d) The percentage of B16F10 cells with positive p-STAT3 immunostain in each group of mice (\(n=3\) /group) was determined. *\(P < 0.02\) versus WT group.
HO activity than their counterparts (Fig. S4b). When the in vitro migration assay was performed, splenic WT-CD11b⁺ cells exhibited greater migration responses toward S100A8, vascular endothelial growth factor (VEGF), and monocyte chemoattractant protein-1 (MCP-1). Nevertheless, the impaired migration response of HO-1⁺/⁻/C0 myeloid cells toward S100A8 could be reversed by cotreatment with a CO donor, tricarbonyldichlororuthenium (II) dimer (CORM-2), but not by the inactive CORM-2 (iCORM-2). In parallel with the migration response, S100A8-induced transient p38 kinase phosphorylation was also much lower in HO-1⁺/⁻ BMDM compared to WT counterparts (Fig. 5c).

**Myeloid Heme oxygenase-1-induced vascular endothelial growth factor and interleukin-10 promote tumor extravasation and STAT3 signaling.** The vascular permeability is crucial for tumor cell extravasation at the metastatic site. VEGF, a potent inducer of endothelial permeability, is induced by HO-1 in macrophages. We confirmed the previous finding that VEGF gene expression was significantly higher in WT-BMDM than HO-1⁺/⁻ BMDM (Fig. S5). To test whether macrophage-
derived VEGF impacts tumor cell extravasation, we performed an in vitro transendothelial migration assay. As shown in Figure 6(a), WT BMDM-CM induced significantly greater transendothelial migration of CFDA-labeled B16F10 cells than HO-1−/− BMDM-CM. Cotreatment with VEGF neutralizing antibody, but not control IgG, resulted in the reduction of increased transendothelial migration of tumor cells induced by BMDM-CM (Fig. 6b). These observations support the involvement of HO-1-induced VEGF in macrophage-mediated tumor cell extravasation at the metastatic site.

To examine whether macrophage-derived soluble factors also contribute to the induction of survival signaling in tumor cells arrested at metastatic sites, we treated B16F10 cells with BMDM-CM for various durations and the level of STAT3 phosphorylation was determined by western blot analysis. The results showed that both WT and HO-1−/− BMDM-CMs induced a rapid and transient phosphorylation of STAT3 (Fig. 6c). In contrast, the level of phospho-STAT3 was much greater in cells treated with WT-BMDM-CM. Along with VEGF, WT-BMDM also expressed higher IL-10 expression comparing to HO-1−/− BMDM (Fig. S5). Considering that both VEGF and IL-10 are potent activators of STAT3, a survival signal for tumor cells, when we performed phospho-STAT3 immunofluorescence staining with lung sections from WT and HO-1−/− mice subjected to i.v. infusion of B16F10 cells, the results showed that the positive phospho-STAT3 stain could be detected on the tumor cells retained in the lungs, and it was much more prominent in WT mice. Notably, these phospho-STAT3+ tumor cells were in close proximity with macrophages, suggesting a possible relevance between macrophages and tumor STAT3 activation in the metastatic foci. This notion was further supported by the in vitro experiment showing that WT-BMDM-CM induced a transient STAT3 phosphorylation of B16F10 cells to a greater extent compared to HO-1−/− BMDM-CM. Moreover, co-treatment of WT-BMDM-CM with neutralizing antibodies against VEGF and IL-10 significantly attenuated the induction of STAT3 phosphorylation in B16F10 cells. These data support a role of HO-1 as a macrophage-mediated survival signal for metastatic tumor cells.

Discussion

In the present study, we demonstrated that HO-1 has a profound effect on the prometastatic function of myeloid cells. Our data show that the migration response of CD11b+ myeloid cells toward S100A8, one of the major chemotactants implicated in the recruitment of myeloid cells to premetastatic lungs, was significantly affected by HO-1 expression via modulating S100A8-induced p38 signaling. This finding is consistent with our early report showing that HO-1 impacts the migration of macrophages to adipose tissue during obesity.(24) Moreover, the impaired migration response of HO-1−/− CD11b+ cells could be reversed by cotreatment with a CO donor. As numerous studies have documented the role of CO in mediating various effects of HO-1,(27) our data support that HO-1/CO-modulated signaling has an impact on the recruitment of myeloid cells to form the premetastatic niche primed by tumor-derived soluble factors.

Experiments also demonstrated that significantly more monocytes/macrophages were recruited to lungs of WT mice in the early phase post-i.v. infusion of B16F10 cells. The percentage of B16F10 cells localized in the extravascular compartments was much higher in WT mice at this time point. This observation is in agreement with early reports showing that the newly recruited macrophages facilitated tumor cell extravasation, a crucial step in metastatic seeding.(9,10) It has been shown that monocyte/macrophage-derived VEGF contributes to the metastatic seeding of breast tumor in lungs.(10) Because HO-1 induces VEGF expression in macrophages, the role of myeloid HO-1-induced VEGF in modulating tumor cell extravasation was supported by the experiment showing that WT-BMDM-CM promoted greater transendothelial migration of tumor cells compared to that of HO-1−/− BMDM, and it could be significantly suppressed by treatment with VEGF neutralizing antibody. These findings indicate that myeloid HO-1 can impact metastatic seeding of tumor cells through VEGF induction.

Whether tumor cells can survive in the foreign microenvironments following extravasation is crucial for successful colonization and growth. Several cytokines and growth factors produced by macrophages, including HO-1-induced VEGF and IL-10, are potent activators of STAT3, a survival signal for tumor cells. When we performed phospho-STAT3 immunofluorescence staining with lung sections from WT and HO-1−/− mice subjected to i.v. infusion of B16F10 cells, the results showed that the positive phospho-STAT3 stain could be detected on the tumor cells retained in the lungs, and it was much more prominent in WT mice. Notably, these phospho-STAT3+ tumor cells were in close proximity with macrophages, suggesting a possible relevance between macrophages and tumor STAT3 activation in the metastatic foci. This notion was further supported by the in vitro experiment showing that WT-BMDM-CM induced a transient STAT3 phosphorylation of B16F10 cells to a greater extent compared to HO-1−/− BMDM-CM. Moreover, co-treatment of WT-BMDM-CM with neutralizing antibodies against VEGF and IL-10 significantly attenuated the induction of STAT3 phosphorylation in B16F10 cells. These data support a role of HO-1 as a macrophage-mediated survival signal for metastatic tumor cells.

In sum, the present study provides the first line of evidence to demonstrate that myeloid HO-1 can facilitate tumor metastasis through promoting premetastatic niche formation and increasing tumor colonization at the metastatic site. Earlier studies have supported the crucial role of NK cells in inhibiting lung metastasis.(28–30) HO-1 has been shown to suppress NK cell-mediated cytotoxicity.(31) Therefore, the contribution of HO-1-induced NK suppression to the enhanced metastasis observed in WT mice cannot be ruled out in the present study. In any event, the design of a therapeutic strategy to specifically downregulate HO-1 expression or its activity in myeloid population may be effective for treating metastatic diseases.

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Disclosure Statement

The authors have no conflict of interest to declare.
Table S1. Vascular endothelial growth factor (VEGF) and interleukin-10 (IL-10) expressions are up-regulated in wild type (WT) macrophages.

Fig. S4. Effect of hematopoietic heme oxygenase-1 (HO-1) haploinsufficiency on HO-1 expression and activity.

Fig. S5. Myeloid HO-1 promotes cancer metastasis.