Reciprocal activation of macrophages and breast carcinoma cells by nitric oxide and colony-stimulating factor-1

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Induction of inducible nitric oxide synthase (iNOS) gene expression, nitric oxide (NO) production and migration of RAW264.7 macrophages by coculture with breast cancer MDA-MB-231 cells or the addition of conditioned medium derived from MDA-MB-231 cells (MDA-CM) was identified. Increased iNOS/NO induction and migration of macrophages by MDA-CM were significantly blocked by adding the c-Jun-N-terminal protein kinase (JNK) inhibitor, SP600125, the nuclear factor-kappa B (NF-kB) inhibitor, BAY117082 and pyrrolidine dithiocarbamic acid and a dominant-negative JNK. The addition of an NO donor, Diethylenetriamine-NONOate, significantly activated expressions of MMP-9 and VEGF-A genes in breast carcinoma MDA-MB-231 cells and invasion of MDA-MB-231 cells in coculture with RAW264.7 macrophages as determined using Transwell systems, but that was inhibited by adding SP600125, BAY117082 and the nitric oxide synthase inhibitor, NG-nitro-L-arginine methyl ester. Induction of heme oxygenase-1 in macrophages reduced MDA-CM-induced iNOS/NO, JNK and NF-kB activations in accordance with inhibiting VEGF-A and MMP-9 gene expressions by MDA-MB-231 cells via Transwell assays. Furthermore, VEGF, sRANKL, TNF-α, IL-1α, TGF-β, CSF-1 and MCP-1 were applied, and CSF-1 showed the most potent stimulations of iNOS/NO production and migration of macrophages. Neutralization of CSF-1 in MDA-CM using CSF-1 antibody inhibited MDA-CM-induced iNOS protein expression and migration of macrophages, and CSF-1-induced iNOS protein and migration was blocked by adding JNK inhibitor SP and NF-kB inhibitor BAY. The reciprocal activation of breast cancer and macrophages via NO-CSF-1 is first elucidated herein.

Introduction

Macrophages play a vital role in immune responses, dealing with pathogenic challenges and wound repair in normal tissues. However, it is well known that macrophages ‘educated’ by a solid tumor may turn into tumor-associated macrophages (TAMs), which promote tumor growth, migration, invasion and metastasis (1,2). Clinical investigations demonstrated that high TAM density present in tumor masses is correlated with a poor prognosis for breast, cervical, bladder and lung cancers (3–6) and decreasing the TAM number reduces the metastatic possibility in mice (7). Tumor cells can release chemok-tractants to recruit macrophages, and activated macrophages then cause the upregulation of an array of proangiogenic and prometastatic factors such as matrix metalloproteinases (MMPs), epidermal growth factor, transforming growth factor (TGF)-β and vascular endothelial growth factor (VEGF) (8–10). In addition, production of proinflammatory molecules such as cyclooxygenase-2 and inducible nitric oxide synthase (iNOS) by TAMs also participate in tumor progression (11). Previous studies demonstrated a paracrine loop via secretion of colony-stimulating factor (CSF)-1 and epidermal growth factor between tumors and macrophages that contributes to the promotion of the invasiveness of breast cancer (12). Li et al. (13) recently demonstrated that TAM-derived iNOS/nitric oxide (NO) regulates the stability of hypoxia-inducible factor-1 and increases vascular survival of tumor cells after exposure to irradiation (13). Although the relationship between tumors and TAMs has been mentioned, evidence regarding the role and detailed mechanism underlying TAMs’ promotion of tumor progression is less well known.

NO, which is liberated from nitric oxide synthase (NOS), mediates several physiologic and pathologic processes. Three isoforms of NOS, endothelial NOS, neuronal NOS and iNOS, have been identified. Large amounts of data indicate a positive correlation of either endothelial NOS or iNOS activity with cancer progression. NO derived from endothelial NOS participates in blood vessel permeability, tumor vascularization and lymph node metastasis (14,15). In addition, expression of iNOS in tumor cells can trigger genetic instability (16,17), induce the expressions of MMP-1 and -2 and VEGF-C and -D and is associated with tumor growth, invasion and lymphangiogenesis (18–20). Stromal cells surrounding tumors including macrophages and fibroblasts can also produce iNOS/NO. The role of iNOS/NO in TAMs’ contribution to tumorigenesis, however, is still unclear.

Recent studies identified a powerful antioxidative stress and anti-inflammatory protein, heme oxygenase (HO)-1, which protects cells against a variety of challenges such as ultraviolet irradiation, free radicals, heavy metals and bacterial infection. Induction of HO-1 was reported to be able to reduce intracellular peroxide levels and suppress the expression of inflammation-associated molecules such as iNOS, tumor necrosis factor (TNF)-α and MMP-9 (21). Beneficial activities of HO-1 were identified in macrophages and endothelial and neuronal cells (22,23). Whether the expression of HO-1 in TAMs contributes to breast cancer, however, is still unknown. Breast cancer exhibits a high propensity to metastasize, and Goswami et al. (24) indicated that macrophages play an important role in promoting the invasion of breast carcinoma cells. However, the contribution of NO in the interaction between macrophages and breast carcinoma cells is still undefined. MDA-MD-231 is a poorly differentiated human breast adenocarcinoma cell line that exhibits aggressive tumorigenic activity in vivo. Coculture of RAW264.7 macrophages with MDA-MB-231 cells using a Transwell coculture system provides evidence that iNOS/NO derived from activated macrophages stimulated the expressions of VEGF-A, MMP-9 messenger (mRNA) and invasion by breast carcinoma MDA-MB-231 cells. The inhibitory effects of HO-1 and the roles of the c-Jun-N-terminal protein kinase (JNK)-nuclear factor-kappa B (NF-kB) cascade in iNOS/NO expression of macrophages activated by breast carcinoma cells were investigated.

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Materials and methods

**Chemicals**

Antibodies against HO-1, iNOS, inhibitor of nuclear factor-kappa B (IkB) α, JNK and CSF-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). α-Tubulin was purchased from Lab Vision (Fremont, CA). Phospho-JNK, p65, phospho-p42/p44 MAPK and phospho-p38 were from Cell Signaling Technology (Beverly, MA). PD98059, SP600125 and SB203580 were obtained from Calbiochem (La Jolla, CA). BAY117082 and DETA-NONOate were from Tocris Cookson (Ellisville, MO). Ferric protoporphyrin IX (FePP) and tin protoporphyrin IX (SnPP) were from Porphyrin Product (Logan, UT). N’-nitro-L-argininemethyl ester (L-NAME), pyridoline dithiocarbamic acid, the probe, triton, charit and phosapate inhibitor cocktail were purchased from Sigma (St Louis, MO). Recombinant CSF-1, monocye chemotactic protein-1, soluble receptor activator of nuclear factor-kappa B ligand (sRANKL), TGF-β and interleukin (IL)-1β were purchased from Peprotech (Rocky Hill, NJ). Recombinant VEGF-C was obtained from R&D (Minneapolis, MN).

**Cell culture**

Human breast carcinoma MCF-7 cells were cultured in minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids at 37°C in a humidified incubator containing 5% CO2. MDA-MB-231 cells and mouse RAW264.7 macrophages were maintained in Dulbecco’s modified Eagle’s medium. MDA-EGFP cells that carried the enhanced green fluorescence protein were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 800 μg/ml G418. All culture reagents were purchased from Life Technologies (Gaithersburg, MD).

**Coculture experiments**

MDA-MB-231 cells were plated in six-well culture plates overnight followed by seeding with RAW264.7 cells for a further 24 h, and the cell lysate and medium were collected. In the Transwell coculture system, RAW264.7 cells were seeded onto Transwell inserts (with a 0.4 μm pore size which was permeable for liquid but not cells; Corning, Corning, NY) for 24 h and placed in serum-free medium (SFM) containing 20% MDA-CM, and the bottom well was supplemented with SFM. After 4 h of stimulation, the Transwells were washed twice with SFM and placed onto a new six-well plate where they were seeded with quiescent MDA-MB-231 cells in the lower chambers for a further 18 h, and expression of indicated genes in MDA-MB-231 cells were examined. A detailed diagram of the experimental procedure is given in Figure 3B.

**Conditioned medium preparation and stimulation**

To collect conditioned medium (CM) from breast cancer, 2 × 10^5 of MCF-7 or MDA-MB-231 cells were seeded overnight; the medium was collected, centrifuged at 2000 r.p.m. to separate out the debris and stored at 4°C. MDA-MB-231-EGFP cells (5 × 10^5) were seeded onto the top well of a Transwell insert (8 μm polycarbonate Nucleopore filters; Corning) and the bottom well insert (8 μm polycarbonate Nucleopore filters; Corning) with 20% MDA-CM (vol/vol) or control medium. After 4 h of stimulation, the Transwells were washed twice with SFM and placed onto a new six-well plate where they were seeded with quiescent MDA-MB-231 cells in the lower chambers for a further 18 h, and expression of indicated genes in MDA-MB-231 cells were examined. A detailed diagram of the experimental procedure is given in Figure 3B.

**Transfection**

Expression vectors of the dominant-negative JNK, pCNA3 control vector, HO-1 small interfering RNA (siRNA) and control siRNA (Santa Cruz, Biotechnology, Santa Cruz, CA) were transiently transfected into RAW264.7 cells using the Lipofectamine 2000 transfection reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer’s instructions. After 24 h, transfected cells were used for further experiments.

**Macrophage migration assay**

RAW264.7 macrophages (1 × 10^5) were seeded onto the top well of a Transwell insert (8 μm polycarbonate Nucleopore filters; Corning) and the bottom well was supplemented with 20% MDA-CM (vol/vol) or control medium. After 16 h of stimulation, cells that had not migrated were removed with a cotton swab, and cells that had migrated to the lower surface of the membrane were subjected to Giemsa staining and observed with a light microscope. Cells that had migrated were counted using three randomly selected fields at ×200 magnification.

**Reverse transcription–polymerase chain reaction**

Total RNA was isolated with an RNA extraction kit (Amersham Pharmacia, Buckinghamshire, UK), and the concentration of total RNA was measured spectrophotometrically. RNA (2 μg) was converted to complementary DNA by an RT–PCR Bead kit (Amersham Pharmacia) according to the manufacturer’s protocol. The amplification sequence was 30 cycles of 94°C for 30 s, 56–60°C for 30 s and 72°C for 1 min. The polymerase chain reaction product of each sample was analyzed by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining. The oligonucleotide primer sequences were as follows:

| Gene | Forward strand | Reverse strand |
|------|----------------|----------------|
| VEGF-A | 5'-CTGGCCCTGTCT | 5'-CACACAGGTAG |
| VEGF-C | 5'-ATTGGCGTGGA | 5'-CGGGAGGTTGTGA |
| MMP-9 | 5'-CAGCTCCACCC | 5'-GCCACCTTGCGCC |
| CSF-1 | 5'-GGAGAGTGTTG | 5'-CATGAGGCTAGT |
| GADPH | 5'-GAAGGTCGGTG | 5'-ACGGATTTGGC |

**Measurement of NO**

The extent of NO production was detected with the Griess reagent. Briefly, 100 μl of medium from stimulated RAW264.7 macrophages was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride in water). The absorbance of the mixture was detected with an enzyme-linked immunosorbent assay at an optical density of 530 nm, with sodium nitrite as the standard.

**Western blots**

Cells lysates were prepared by suspending cells in lysis buffer (50 mM Tris–HCl (pH 7.4), 1% Nonidet P-40, 150 mM NaCl, 1 mM ethyleneglycol-bis(aminohexylether)-tetraacetic acid, 0.025% sodium deoxycholate, 1 mM sodium fluoride, 1 mM sodium orthovanadate and 1 mM phenylmethylsulfonyl fluoride). An equal amount of protein was added to 5 × sodium dodecyl sulfate–sample buffer with 2-mercaptoethanol and separated on 8% sodium dodecyl–polyacrylamide gels. The western blot procedure was carried out as described previously (25).

**Invasion assay**

The invasion assay was carried out using a 24-well Transwell unit with 8 μm polycarbonate Nucleopore filters (Corning) coated with 60 μl of 0.8 mg/ml Engelbreth-Holm-Swarms carcinoma tumor extract (EHS Matrigel) at room temperature for 2 h to form a genuine reconstituted basement membrane. MDA-MB-231-EGFP cells (5 × 10^5) and RAW264.7 macrophages (2 × 10^5) were placed in the upper compartment, and CM was added to the lower compartment. The Transwell plates were incubated at 37°C for 36 h, and cells that had not invaded were removed with a cotton swab. Cells that had invaded the lower surface of the membrane were observed and pictured using a fluorescence microscope. Invaded cells in three randomly selected fields were counted at ×40 or ×100 magnification.

**CSF-1 neutralization**

CM (200 μl) derived from MDA-MB-231 was incubated with or without different concentrations (1 and 2 μg) of anti-CSF-1 antibodies and incubation at 4°C for 16 h, followed by adding A plus G bead for an additional 1 h after centrifugation at 2000g for 10 min, the supernatant was collected for migration assay by Transwell assay or detection of iNOS protein expression by western blotting in RAW264.7 macrophages.

**Measurement of CSF-1 secreted from MDA-MB-231 and MCF-7 cells**

MDA-MB-231 or MCF-7 cells were seeded in 24-well plate and incubated at 37°C for 24 h. The CM was collected and centrifuged at 3000 r.p.m. for 5 min to remove the pellet. CSF-1 in the supernatant was measured with enzyme-linked immunosorbent assay (Quantikine Human CSF-1 Immunoassay, DY216; R&D systems), according to the manufacturer’s instructions. This assay employs the quantitative sandwich enzyme immunoassay technique, and the minimum detectable dose of CSF-1 is typically <7.8 pg/ml. The assay does not exhibit cross-reactivity with series of cytokines and growth factors.

**Statistical analyses**

Values are expressed as the mean ± SE. The significance of the difference from the respective controls for each experimental test condition was assessed using Student’s t-test for each paired experiment. A P value of <0.01 or <0.05 was regarded as indicating a significant difference.
Results

Stimulation of iNOS/NO and migration of RAW264.7 macrophages by CM derived from breast carcinoma MDA-MB-231 cells (MDA-CM)

As shown in Figure 1A, increases in iNOS protein and NO production were detected with the coculture of RAW264.7 macrophages and MDA-MB-231 cells, but not in macrophages or MAD-MB-231 cells alone. Elevation of NO production by lipopolysaccharide (LPS) plus interferon-γ (L/I) in macrophages was used as a positive control. To elucidate which cells contribute to iNOS/NO activation in the coculture system, CMs derived from control, MDA-MB-231 (MDA-CM), and RAW264.7 cells were prepared as described in ‘Materials and Methods’. As shown in Figure 1B, an increase in iNOS/NO activation was detected in MDA-CM-treated RAW264.7 macrophages. However, CM from RAW264.7 showed no effect on iNOS/NO expression in MDA-MB-231 cells (data not shown). Furthermore, the migration of RAW264.7 macrophages elicited by MDA-CM was examined using Transwell assays. As illustrated in Figure 1C, a significant induction of the migration of RAW264.7 macrophages by MDA-CM was identified by elevated NO production. This suggests that breast cancer MDA-MB-231 cells may activate migration and iNOS/NO production in macrophages.

MDA-CM induces iNOS/NO production in macrophages via activating JNK and NF-κB

Pharmacological studies using specific inhibitors were performed to elucidate the mechanism of iNOS/NO production elicited by

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**Fig. 1.** CM from breast cancer MDA-MB-231 cells induces iNOS/NO production and macrophage migration in RAW264.7 cells. (A) An increase in iNOS protein in coculture of MDA-MB-231 cells with RAW264.7 cells. Lane 1, human breast cancer MDA-MB-231 cells (3 x 10^5; MDA); lane 2, RAW264.7 macrophages (3 x 10^5; Mac); lane 3, coculture of MDA-MB-231 and RAW264.7 cells (MDA + Mac). Mac + L/I indicated that cells were treated with LPS plus interferon-γ as a positive control. iNOS protein expression and NO production were detected by western blotting (left) and the Griess reagent (right), respectively. (B) Induction of iNOS/NO production by adding CM [MDA-CM; 20% (vol/vol)] from MDA-MB-231 cells. RAW264.7 cells were treated with 20% MDA-CM for 24 h, and the expression of iNOS protein and NO production were determined by western blotting (left) and the Griess reagent (right), respectively. (C) MDA-CM induction of the migration of RAW264.7 cells via a Transwell assay. RAW264.7 cells were seeded in the top well, and MDA-CM or Con-CM (CON) medium after incubation at 37 °C overnight in the condition without MDA-MB-231 cells was added to the lower wells for 16 h. The migration of RAW264.7 cells was analyzed as described in ‘Materials and Methods’ (bar = 100 μm). Measurement of NO production in the upper well was evaluated by the Griess reagent. Data are expressed as the mean ± SE from three independent experiments by Student’s t-test. *P < 0.05 and **P < 0.01 denote a significant difference between indicated groups (A) or compared with the CON group (B and C).
MDA-CM. As shown in Figure 2A and B, the MDA-CM-induced iNOS protein was inhibited by adding JNK, but not the extracellular signal-regulated kinases or p38 inhibitor, SP600125, with a reduction of JNK protein phosphorylation in RAW264.7 cells. Transfection of the dominant-negative mutant of JNK into macrophages significantly reduced iNOS/NO production in macrophages elicited by MDA-CM (Figure 2C). Additionally, activation of NF-κB including cytosolic phosphorylated IκBα and nuclear p65 proteins increased in RAW264.7 cells stimulated by MDA-CM (Figure 2D). However, MDA-CM-induced phosphorylations of IκBα and nuclear p65 protein were blocked by adding SP600125, but not PD98059 or SB203580 (Figure 2E). Application of a specific IκBα inhibitor, BAY117082, dose-dependently inhibited iNOS/NO production induced by MDA-CM (Figure 2F). Additionally, NF-κB inhibitor pyrrolidine dithiocarbamic acid addition suppressed MDA-CM-induced iNOS/NO production and migration in RAW264.7 macrophages (Figure 2G). This suggests that MDA-CM-induced iNOS/NO production is mediated by activation of the JNK–NF-κB cascade in macrophages.

**Fig. 2.** Activation of the JNK/NF-κB pathway in MDA-CM-induced iNOS/NO expression in macrophages. (A) The JNK inhibitor, SP600125, but not extracellular signal-regulated kinases or p38 inhibitors, reduced MDA-CM-induced iNOS protein expression in macrophages. Macrophages were treated with different concentrations (10 and 20 μM) of PD98059 (PD), SP600125 (SP) or SB203580 (SB) for 30 min followed by 20% MDA-CM stimulation for an additional 24 h. iNOS and α-tubulin (α-Tub) protein expression in each group was analyzed by western blotting. (B) Inhibition of MDA-CM-induced JNK protein phosphorylation by SP600125. Cells were treated with different concentrations of SP600125 for 30 min followed by MDA-CM stimulation for an additional 30 min. Expression of phosphorylated and total JNK protein was examined by western blotting using specific antibodies. (C) Transient transfection of macrophages with the dominant-negative c-Jun-N-terminal protein kinase (DN-JNK) plasmid blocked MDA-CM-induced iNOS protein expression and NO production by RAW264.7 cells. The vector or DN-JNK (1 ng)-transfected macrophages were treated with 20% MDA-CM for 24 h, and iNOS protein and NO levels were determined. (D) MDA-CM induction of IκB protein phosphorylation and nuclear p65 protein in RAW264.7 macrophages. RAW264.7 cells were incubated with MDA-CM (20%), and the expressions of phospho-IκB, -p65 and total-IκB were analyzed by western blotting. (E) The JNK inhibitor, SP600125 (SP), but not PD or SB, blocked MDA-CM-induced cytosolic phospho-IκB and nuclear p65 protein in RAW264.7 macrophages. Macrophages were treated with PD, SP or SB (20 μM) for 30 min followed by 20% MDA-CM stimulation for an additional 60 min. Expression of cytosolic phospho-IκB and nuclear p65 were examined, and no change in α-Tub or the Poly (ADP-ribose) polymerase protein was used as the respective cytosolic and nuclear internal controls. (F) The NF-κB inhibitor, BAY117082, suppressed MDA-CM-induced iNOS protein and NO production by RAW264.7 macrophages. Macrophages were treated with different concentrations of the IκB inhibitor, BAY117082 (BAY; 5, 10 and 20 μM), for 30 min followed by 20% MDA-CM stimulation for an additional 60 min. Expression of cytosolic phospho-IκB and nuclear p65 were examined, and no change in α-Tub or the Poly (ADP-ribose) polymerase protein was used as the respective cytosolic and nuclear internal controls. (G) PDTC inhibition of MDA-CM-induced iNOS/NO production and migration in macrophages. Upper panel: cells were treated with different concentrations (0.5, 1 and 2 mM) of PDTC for 30 min followed by 20% MDA-CM stimulation for 24 h. Expression of iNOS protein was examined by western blotting. Lower panel: as described before, PDTC (1 mM) inhibition of migration and NO production stimulated by 20% MDA-CM in macrophages. Data are expressed as the mean ± SE from three independent experiments. *P < 0.01 indicates a significant difference between the indicated groups (C) or compared with the MDA-CM-treated group (F and G) by Student’s t-test.
The NO donor, Diethylenetriamine-NONOate and macrophage coculture induce VEGF-A and MMP-9 gene expressions and invasion by breast carcinoma MDA-MB-231 cells

We further examined the role of NO released by macrophages in the invasiveness of breast carcinoma MDA-MB-231 cells. First, MDA-MB-231 cells were treated with a slow NO-releasing donor (DETA-NONOate) and expressions of metastatic genes including VEGFs, VEGF-c, VEGF-A and MMP-9 at the mRNA level were analyzed by reverse transcription–polymerase chain reaction. As shown in Figure 3A, increases in VEGF, VEGF-A and MMP-9 mRNA expressions with NONOate stimulation were observed; however, the mRNA level of VEGF-C was not altered. To provide evidence that NO from macrophages contributes to metastatic gene expressions in MDA-MB-231 cells, a coculture system as shown in Figure 3B was used. Briefly, macrophages in the upper chamber were treated with MDA-CM with or without the indicated inhibitors for 4 h, and cells were washed twice with phosphate-buffered saline and incubated in fresh Dulbecco’s modified Eagle’s medium and placed into a new Transwell that contains MDA-MB231 cells in the lower chamber. After 18 h, expressions of VEGF-A and MMP9 mRNA in MDA-MB-231 cells were detected. As illustrated in Figure 3C, the mRNA expressions of VEGF-A and MMP9 were induced in MDA-MB-231 cells cocultured with macrophages. In the presence of the NOS inhibitor, L-NAME, in RAW264.7 macrophages, increased VEGF-A and MMP-9 mRNA expressions in MDA-MB-231 cells stimulated by macrophage coculture were blocked (Figure 3D). Accordingly,
pretreatment of RAW264.7 cells with SP600125 and BAY117082 suppressed VEGF-A and MMP-9 mRNA expressions by MDA-MB-231 cells (Figure 3E). These data suggest that NO derived from activated macrophages contributes to VEGF-A and MMP9 mRNA expressions in breast carcinoma cells. We further examined if coculturing macrophages and breast carcinoma cells could stimulate invasion by breast carcinoma cells. Green fluorescent protein (GFP)-expressing MDA-MB-231 cells (MDA-EGFP) were cocultured with RAW264.7 cell coculture, and it was blocked by SP600125, BAY117082 and L-NAME (Figure 3F).

**Induction of HO-1 reduces iNOS/NO production in macrophages elicited by MDA-CM**

HO-1 was shown to inhibit iNOS/NO production in macrophages elicited by LPS or lipoteichoic acid in our previous studies (26,27). Therefore, we investigated if HO-1 can reduce iNOS/NO production in macrophages stimulated by MDA-CM. As shown in Figure 4A, the HO-1 inducer, FePP, induced HO-1 protein with attenuation of iNOS protein expression and NO production elicited by MDA-CM (Figure 4A). MDA-CM-induced JNK and IκB protein phosphorylations were reduced by FePP addition with an increase in HO-1 protein expression (Figure 4B). Suppression of FePP-induced HO-1 protein by HO-1 siRNA, but not control siRNA, attenuated the inhibitory effect of FePP against MDA-CM-induced iNOS/NO production in macrophages (Figure 4C).

**FePP inhibition of MDA-CM-induced NO production was reversed by adding the HO-1 inhibitor, SnPP (Figure 4D).**

**Induction of HO-1 in macrophages reduced VEGF-A and MMP-9 expressions and tumor invasion**

We further assessed if induction of HO-1 in macrophages could reduce the invasiveness of breast cancer MDA-MD-231 cells. As shown in Figure 5A, FePP incubation inhibited NO production by RAW264.7 macrophages with a decrease in VEGF-A and MMP-9 gene expressions in MDA-MB-231 cells with the Transwell coculture system (Figure 5A and B). Data from the in vitro invasion assay indicated that FePP treatment of RAW264.7 macrophages significantly inhibited the invasion of MDA-EGFP cells in the coculture system (Figure 5C). These data suggest that induction of HO-1 by macrophages can inhibit the invasion of breast carcinoma MDA-MB-231 cells via blocking iNOS/NO production by macrophages.

**Involvement of CSF-1 in iNOS and migration of macrophages**

We further examined which factors released by breast carcinoma MDA-MB-231 cells contribute to iNOS induction and migration of macrophages. As illustrated in Figure 6A, CSF-1, but not VEGF-C, sRANKL, TNF-α, IL-1α, TGF-β or monocyte chemotactic protein-1, increased iNOS protein expression by RAW264.7 macrophages. Data of the migration assay showed that CSF-1 shown the most potent induction on the migration of RAW264.7 macrophages as did MDA-CM (Figure 6B). MCF-7 is a non-invasive breast cancer cell line, and a lower level of CSF released from MCF-7 compared with that released from MDA-MB231, via enzyme-linked immunosorbent
assay (Figure 6C). Analysis of CSF-1 mRNA expression in both breast carcinoma cells by reverse transcription–polymerase chain reaction using specific primers showed that a lower level of endogenous CSF-1 mRNA expression in MCF-7 cells than that in MDA-MB-231 cells (Figure 6D). CM derived from MCF-7 showed less of an inductive effect on iNOS protein expression than MDA-CM (Figure 6E). Similarly, CM from MCF-7 was less effective at inducing the migration of RAW264.7 macrophages compared with MDA-CM (Figure 6F). Addition of JNK inhibitor SP500125 (SP) and NF-κB inhibitor BAYE significantly reduced CSF-1-induced iNOS and p-JNK protein expression and migration in macrophages (Figure 6G). Neutralization of CSF-1 in MDA-CM using anti-CSF-1 antibody inhibited iNOS protein expression and migration of RAW264.7 macrophages (Figure 6H). This suggests that CSF-1 is involved in macrophage activation by breast carcinoma cells.

Discussion

We show that factors in MDA-CM employ JNK and NF-κB activation to stimulate iNOS/NO activation and the migration of macrophages. We further identified that NO derived from MDA-CM-activated macrophages induces invasion by breast carcinoma MDA-MB-231 cells with concomitant increases in VEGF-A and MMP-9 gene expressions. HO-1 induction in macrophages reduced iNOS/NO expression and migration of macrophages, and NO derived from activated macrophages contributes to invasion by breast carcinoma cells. A reciprocal interaction of breast carcinoma cells and macrophages via NO and CSF-1 was indicated.

Macrophages play a critical role in the progression of malignant tumors and in immune surveillance against established tumors. It has been recognized that a direct correlation between the quantity of TAMs and a poor prognosis. Two types of macrophages including M1 and M2 have been proposed, and M1 macrophages are typically pro-inflammatory and M2 macrophages promote tumor progression (28,29). NO accumulation occurs in M1 macrophages stimulated by interferon-γ alone or in combination with LPS, and activation of arginase in M2 macrophages promote tumor progression. RAW264.7, a murine macrophage cell line, has been shown to possess ability to stimulate NO production under LPS plus interferon-γ treatment (30). Morris et al. (31) indicated induction of arginase isoforms was detected in RAW264.7 macrophages. It suggests that RAW264.7 macrophages reserve the characteristics of M1 and M2 macrophages. Interaction of murine RAW264.7 macrophages with human or murine tumor cells has been reported in several previous studies. Joshi et al. (32) indicated RAW264.7 macrophages-mediated antibody-dependent cell-mediated cytotoxicity against human Burkitt’s lymphoma cells Raji. Green et al. (33) indicated that RAW264.7 macrophages promoted the migration and invasion of murine colon carcinoma cells CT26. The Alliance for Cellular Signaling generated a systemic profile of cytokines using RAW264.7 macrophages. It suggests that RAW264.7 combined with breast cancer model here may provide new insights into the interaction between macrophages and breast tumor cells.

Disrupting the cooperation between TAMs and the tumor has therapeutic potential for cancer treatment. One strategy is to target TAMs-derived biomarkers, thereby cutting off communication and the trend

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Fig. 5. HO-1 induction in macrophages inhibited VEGF-A and MMP-9 expressions and invasion by breast carcinoma MDA-MB-231 cells. (A) FePP inhibition of NO production in the presence of RAW264.7 macrophages and breast carcinoma MDA-MB-231 cells. RAW264.7 cells were cocultured with MDA-MB-231 cells in the presence of FePP (20 μM) as described previously, and the amount of NO in the medium was measured by the Griess reagent. *P < 0.01 indicates a significant difference between the indicated groups. (B) Left panel: FePP inhibited the expressions of VEGF-A and MMP-9 mRNA in breast carcinoma MDA-MD-231 cells in the coculture system as described in (A). Right panel: VEGF-A and MMP-9 band intensities were quantified by ImageJ software and are represented as multiples of the control. (C) FePP inhibited the invasion by breast carcinoma MDA-MB-231 cells when cocultured with macrophages. RAW264.7 cells treated with or without FePP (20 μM) were cocultured with MDA-EGFP cells in Matrigel-coated Transwell inserts for 36 h, and MDA-MD-231 cells that had invaded were detected by fluorescence microscopy at ×100 magnification (bar = 500 μm). Right panel: the number of invaded cells was quantified, and data are expressed as the mean ± SE from three independent experiments by Student’s t-test.
toward cancer development (34,35). In the present study, we show that induction of iNOS/NO by activated macrophages plays an important role in promoting the invasion of MDA-MB-231 cells. MDA-MB-231 cells were able to activate macrophages with induction of iNOS/NO production and migration, and NO released by activated macrophages stimulated the invasion of MDA-MB-231 cells by elevating VEGF-A and MMP-9 expressions. Suppression of NO production by an NOS inhibitor or HO-1 in macrophages significantly reduced the invasiveness of breast carcinoma cells in the coculture system. A beneficial effect of NO inhibition of preventing the invasion by breast carcinoma cells was demonstrated.

Treatment of macrophages with inducers such as LPS, Phorbol 12-myristate 13-acetate, IL-4, IL-13 and colon cancer-derived CM stimulates the activation and migration of macrophages (10,36–38), and activated TAMs enhance the metastatic behavior of several tumors such as colon carcinoma, melanoma, lung and breast cancers (10,37,39,40). Yao et al. (41) indicated that lung cancer causes macrophages to produce such inflammatory cytokines as TNF-α and IL-1α,

Fig. 6. CSF-1 stimulation of iNOS protein expression and migration of RAW264.7 macrophages. (A) CSF-1 but not other factors stimulated iNOS protein expression in RAW264.7 cells. Cells were treated with different concentrations of the indicated chemokines or growth factors for 36 h, and the expression of iNOS protein was determined by western blotting. CSF-1 (0–200 ng/ml), VEGF-C (0–200 ng/ml), sRANKL (0–100 ng/ml), TNF-α (0–100 ng/ml), IL-1α (0–50 ng/ml), TGF-β (0–100 ng/ml) and monocyte chemotactic protein (MCP)-1 (0–100 ng/ml). (B) CSF-1 but not other factors induced the migration of RAW264.7 macrophages. Macrophages were treated with the indicated molecules for 36 h in the Transwell assay, and cells that had migrated were counted. (C) Amount of CSF-1 released in the CM of MCF-7 and MDA-MB-231 cells. Both cells in different cell numbers (2.5 × 10⁵ and 5 × 10⁵) were seeded and incubated in 37°C for 36 h. Amount of CSF-1 in the medium derived from MCF-7 and MDA-MB-231 cells were measured by enzyme-linked immunosorbent assay. (D) Expression of CSF-1 mRNA is high in breast carcinoma MDA-MB-231 cells and low in MCF-7 cells, by reverse transcription–polymerase chain reaction assay. mRNA derived from both types of cell was prepared to detect CSF-1 mRNA expression by reverse transcription–polymerase chain reaction using specific primers. (E) CM from MDA-MD-231 cells induced greater iNOS protein expression than that from MCF-7 cells. CM from both cells was prepared as described before, and RAW264.7 cells were treated with 20% CM for 24 h; the expressions of iNOS and α-tubulin protein were examined by western blotting. (F) CM from MDA-MB-231 cells was more effective in inducing the migration of RAW264.7 macrophages than that from MCF-7 cells. As described in (B), the migration of macrophages with 20% CM from each cell type was analyzed by a Transwell assay. (G) JNK inhibitor SP600125 (SP) and NF-κB inhibitor BAY inhibits CSF-induced iNOS and JNKs protein expression and migration of RAW264.7 macrophages. Cells were treated with SP (20 µM) or BAY (10 µM) for 30 min followed by CSF-1 (200 ng/ml) stimulation for 36 h (iNOS protein and migration) or 30 min (JNK protein). Upper panel: expression of iNOS, p-JNK, total-JNK and α-tubulin (α-Tub) protein was analyzed by Western blotting. Lower panel: migration of macrophages under different treatments was examined by a Transwell assay. (H) Neutralization of CSF-1 using anti-CSF-1 antibodies reduced iNOS protein expression and migration stimulated by MDA-CM in RAW264.7 macrophages. As described in the section of Materials and Methods, expression of iNOS protein (upper panel) and migration (lower panel; CSF-1, 1 µg) of macrophages under different stimulations was examined. Data are expressed as the mean ± SE from three independent experiments. *P < 0.01, **P < 0.01 indicate a significant difference compared with the CON group (B and F) or CSF-1-treated group (G and H) by Student’s t-test.
which further stimulates angiogenesis of lung cancer. Although the contribution of activated macrophages to tumor invasion was addressed, little is known about the mechanism involved in macrophage activation. In the present study, activation of JNK and NF-kB in RAW264.7 macrophages by MDA-CM or coculture with MDA-MB-231 cells was detected, and macrophage activation elicited by MDA-MB-231 cells was blocked by inhibitors of JNK or NF-kB. This suggests that activation of JNK and NF-kB is involved in macrophage activation stimulated by breast carcinoma MDA-MB-231 cells.

NO was demonstrated to be an important mediator during inflammation, but its relevance in tumors remains paradoxical as it participates in both tumorigenesis and tumoridical activities. Recent studies reported that the expression of iNOS in either tumor cells or TAMs may eventually contribute to the growth of tumors. Positive correlations of iNOS with the malignant grade of breast, head and neck and colorectal cancers were noted (42,43). Treatment with a slow-release NO donor (DETA-NONOate) increased the proliferation of breast cancer MDA-MB-231 and MCF-7 cells (44), and the iNOS inhibitor, 1400W, decreased the mobility and survival of murine and human mammary tumor cells (45), indicating that NO participates in tumor progression. However, NO is also involved in the macrophage-mediated cytotoxicity of tumor cells (46). Although a role of NO in tumor progression was reported, mechanisms by which NO promotes tumor invasion are not fully understood. Herein, coculture of activated macrophages and breast carcinoma MDA-MB231 cells induced invasion by MDA-MB-231 cells with increases in VEGF-A and MMP-9 gene expressions, which were prevented by adding the iNOS inhibitor, L-NAME. The addition of a slow NO-releasing donor, DETA-NONOate, significantly induced invasion by breast carcinoma cells with elevated VEGF-A and MMP-9 gene expressions. The involvement of NO in the invasion by breast carcinoma cells stimulated by activated macrophages and mediated by increased VEGF-A and MMP-9 gene expression was identified.

Beneficial effects of HO-1 such as anti-inflammation and cytoprotection are mentioned in the literature, but whether induction of HO-1 in TAMs plays a role in tumorigenesis is still unclear (21,47). Boschetto et al. (48) showed that HO-1 expression decreased in macrophages of non-small-cell lung cancer compared with tumor-free macrophages, implying impairment of the antioxidant defense systems may be a reason for tumorigenesis. Conversely, Nishie et al. (49) indicated that HO-1 is a marker mediating macrophage infiltration and angiogenesis in glioma cells. We herein showed that HO-1 induction inhibited iNOS/NO production and migration of macrophages, which possibly occurred through diminishing JNK and NF-kB activation, mediated by MDA-CM. Additionally, HO-1 induction in macrophages may suppress VEGF-A and MMP-9 gene expressions by MDA-MB-231 cells in a coculture system. Evidence indicates that HO-1 induction reduced NO production in macrophages and consequently prevented the invasion by breast cancer cells.

Breast carcinoma-derived factors such as parathyroid hormone-related peptide, CSF-1, epidermal growth factor and cytokines were identified, and high levels of CSF-1 in the tumor microenvironment were reported; this attracts TAM infiltration and is correlated with tumor metastasis (33,50). Goswami et al. (24) indicated that breast carcinoma cells are able to release CSF-1 to activate macrophages, which express the CSF-1 receptor, and interfere with CSF-1 receptor’s reduction in the invasion by breast carcinoma cells. The release of CSF-1 by breast cancer cells also cooperates with rSANKL in osteoclasts during tumor bone metastasis (51,52). However, which factor is released by breast carcinoma cells to stimulate iNOS/NO production in macrophages remains undefined. Among seven known factors, CSF-1 showed a significant positive effect on iNOS gene expression, and CSF-1 stimulation of macrophage migration was observed. Additionally, low levels of CSF-1 mRNA in breast carcinoma MCF-7 cells stimulated less iNOS protein and migration of macrophages than those obtained by MDA-MB-231 cells. This suggests that CSF-1, at least in part, is involved in breast cancer-derived factors to stimulate iNOS expression and migration of macrophages.

Previous studies have shown that macrophages regulate the angiogenic switch, and CSF-1 secretion by breast cancer cells is a potent chemoattractant for macrophages in vivo (12,24,50). These studies clearly show that tumor-derived CSF-1 can direct macrophages migration and infiltration into the tumor, however, what factor stimulated by CSF-1 in macrophages to promote tumor invasion is still undefined. Our findings indicate that CSF-1 production by breast carcinoma cells MDA-MB-231 induces iNOS/NO production and migration of macrophages. NO released by macrophages activates the expressions of VEGF-A and MMP-9 mRNA and promotes invasion of breast carcinoma cells. Neutralization of CSF-1 in CM derived from MDA-MB-231 cells by anti-CSF-1 antibody significantly reduced iNOS protein expression and migration of macrophages. Based on our findings and the work of others, we propose a hypothetical model of a reciprocal activation between breast carcinoma cells and macrophages via iNOS/NO and CSF-1 leading to an increased carcinoma invasive potential as shown in Figure 7. Because macrophages have a lower propensity to develop multidrug resistance and are genetically stable, it would be beneficial to block breast carcinoma invasion via targeting macrophages activated by cancers. Our study provides evidence that iNOS/NO from macrophages activated by carcinomacanceroma cells contributes to the invasive potential of carcinoma cells. Applications of iNOS inhibitors or NO scavengers may possess clinical benefits in the treatment of breast cancers.

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