Brain-derived neurotrophic factor (BDNF) is a potent neurotrophic factor that has been shown to affect cancer cell metastasis and migration. In the present study, we investigated the mechanisms of BDNF-induced cell migration in colon cancer cells. The migratory activities of two colon cancer cell lines, HCT116 and SW480, were found to be increased in the presence of human BDNF. Heme oxygenase-1 (HO)-1 is known to be involved in the development and progression of tumors. However, the molecular mechanisms that underlie HO-1 in the regulation of colon cancer cell migration remain unclear. Expression of HO-1 protein and mRNA increased in response to BDNF stimulation. The BDNF-induced increase in cell migration was antagonized by a HO-1 inhibitor and HO-1 siRNA. Furthermore, the expression of vascular endothelial growth factor (VEGF) also increased in response to BDNF stimulation, as did VEGF mRNA expression and transcriptional activity. The increase in BDNF-induced cancer cell migration was antagonized by a VEGF-neutralizing antibody. Moreover, transfection with HO-1 siRNA effectively reduced the increased VEGF expression induced by BDNF. The BDNF-induced cell migration was regulated by the ERK, p38, and Akt signaling pathways. Furthermore, BDNF-increased HO-1 and VEGF promoter transcriptional activity were inhibited by ERK, p38, and AKT pharmacological inhibitors and dominant-negative mutants in colon cancer cells. These results indicate that BDNF increases the migration of colon cancer cells by regulating VEGF/HO-1 activation through the ERK, p38, and PI3K/Akt signaling pathways. The results of this study may provide a relevant contribution to our understanding of the molecular mechanisms by which BDNF promotes colon cancer cell motility.
Introduction

Colon cancer is one of the most common gastrointestinal sarcomas and a leading cause of cancer-associated death in developed countries (Siegel et al. 2014). It can be treated by surgery and combination chemotherapy. The etiology of colon cancer remains unclear, but it is believed that environmental factors, genetic factors, and precancerous diseases are associated with the progression of colon cancer (Aarons et al. 2014). Colon cancer can grow into the lumen or adjacent structures, and this is one of the ways in which the disease develops to advanced colon cancer. Approximately 25% of patients present with metastases at initial diagnosis, and almost 50% of patients with colon cancer will develop metastases, and these contribute to the high mortality rates (Van Cutsem et al. 2010). The metastases of tumors are associated with a patient’s survival, and they account for about 90% of all colon cancer deaths (Mehlen & Puisieux 2006). Early diagnosis and treatment may contribute to improvement in the survival rates and prognostic evaluation of colon cancer.

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family that is widely expressed in the mammalian brain (Binder & Scharfman 2004). BDNF is required for the development and proper functioning of the CNS. It involves in a variety of neural and molecular events and plays an important role in both brain development and synaptic plasticity (Hong et al. 2011). Recently, BDNF has been reported to be associated with tumor progression in several human malignancies, such as neuroblastoma (Czarnecka et al. 2014), lung cancer (Okamura et al. 2012), breast cancer, and colon cancer (Yang et al. 2012, 2013). BDNF and its receptor, TrkB, are commonly upregulated in a variety of human tumors (Guo et al. 2011). Moreover, the overexpression of BDNF and TrkB is related to poor clinical outcome and to reduced survival rates in human breast cancer and bladder cancer (Lai et al. 2010, Patani et al. 2011). The roles played by BDNF/TrkB signaling in breast cancer proliferation and survival have been demonstrated (Ricci et al. 2001). However, the precise biological role of BDNF and its utility as a novel biomarker in colon cancer are still yet to be determined. The objective of the present study was to determine the mechanism that underlies BDNF-regulated cell motility in human colon cancer cells.

Vascular endothelial growth factor (VEGF) is a central regulator of angiogenesis in physiological and pathological conditions. VEGF is upregulated in response to hypoxia, activated oncogenes, and a variety of cytokines (Sismanopoulos et al. 2012, Raja et al. 2014). VEGF stimulates normal epithelial cells and differentiated carcinoma cells by inducing epithelial–mesenchymal transition (EMT) (Bates et al. 2003). It has been shown that VEGF signaling synergizes with epidermal growth factor receptor signaling to promote the development of squamous carcinoma (Lichtenberger et al. 2010). It has also been demonstrated that expression of VEGF and VEGFR via the induction of EMT promotes progression to more aggressive tumors in colon carcinoma cells (Bates et al. 2003). Moreover, increased VEGF expression has been found to correlate with an increased incidence of metastasis and decreased patient survival in colon cancer (Takahashi et al. 1995). VEGF-targeted therapy benefits patients with advanced-stage malignancies in colon cancer (Ellis & Hicklin 2008). During a recent study we determined that VEGF–VEGF receptor interaction promotes colon cancer motility (Huang et al. 2014b). Modulation of VEGF function may contribute to a successful therapeutic treatment of colon cancer and may be applied as a prognostic marker in colon cancer.

In the present study, we investigated the intracellular signaling pathways involved in the BDNF-regulated cell motility in colon cancer cells. Our results indicated that BDNF induced cell migration by upregulating VEGF expression via the induction of heme oxygenase-1 (HO-1). Moreover, BDNF directed the migration of colon cancer cells through the activation of the ERK, p38, and Akt signaling pathways. Therefore, investigation of the molecular mechanism for targeting angiogenesis and migratory pathways in tumors may help in preventing the progression of colon cancer.

Materials and methods

Materials

BDNF was purchased from PeproTech (Rocky Hill, NJ, USA). Tin protoporphyrin (SnPP), PD98059, SP600125, and LY294002 were obtained from Sigma–Aldrich. Cobalt protoporphyrin (CoPP), zinc protoporphyrin IX (ZnPP), K252a, and AKT inhibitor (1L-6-hydroxymethyl-chiro-inositol 2(R)-2-(pyridin-4-ylmethyl)amino) benzamide was purchased from Calbiochem (San Diego, CA, USA). VEGF receptor inhibitor N-(4-chlorophenyl)-2-((pyridin-4-yl)aminomethyl)-3-octadecylcarbonate were purchased from Calbiochem (San Diego, CA, USA). VEGF receptor inhibitor N-(4-chlorophenyl)-2-((pyridin-4-yl)methyl)benzamide was purchased from Merck Co. Fetal bovine serum (FBS), DMEM and OPTI-MEM were purchased from Gibco BRL (Invitrogen Life Technologies). Primary antibody against HO-1 was purchased from StressGen Biotechnologies (Victoria, BC, Canada).
Canada). Primary antibodies against p-ERK1/2, p-Akt ERK2, p38, JNK, Akt, and β-actin were purchased from Santa Cruz Biotechnology. Control siRNA and HO-1 siRNA, a pool of five target-specific siRNAs designed to knock down gene expression, were also purchased from Santa Cruz Biotechnology. P-JNK and p-p38 were purchased from Cell Signaling and Neuroscience (Danvers, MA, USA). A luciferase assay kit was purchased from Promega. The DN mutants of ERK (DN-ERK), p38 (DN-p38), Akt (DN-AKT), and VEGF-luciferase plasmids were gifts from Dr W-M Fu (National Taiwan University, Taipei, Taiwan).

Cell cultures

The human colon carcinoma cell lines HCT-116 and SW480 were obtained from the American Type Culture Collection (Manassas, VA, USA). Short tandem repeat profiles were examined by the Bioresource Collection and Research Center (BCRC, Taiwan) after the present study had concluded to ensure the quality and integrity of these two cell lines. The cell culture protocol was performed as described in our previous publication (Huang et al. 2011). Briefly, cells were grown in plastic cell culture dishes in DMEM, supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin, at 37 °C in a humidified incubator under an atmosphere of 5% CO₂ and 95% air. Passages of HCT-116 cells from 46 to 58 and SW480 cells from 19 to 22 were used in the experiments.

Transmigration assay

An in vitro migration assay was performed using Costar Transwell inserts (Costar, Brooklyn, NY, USA; pore size 8 µm) as described in our previous reports (Chuang et al. 2013, Huang et al. 2014a). Before performing the transmigration assay, cells were pretreated for 30 min with different concentrations of inhibitors or transfected with various DN mutants for 24 h. On the basis of the results of a cell viability assay, the various concentrations of inhibitors used did not affect colon cancer cell death (data not shown). Approximately 1 × 10⁴ cells in 100 µl of serum-free medium were placed in the upper chamber, and 400 µl of the same medium containing BDNF were placed in the lower chamber. The plates were incubated for 24 h at 37 °C in 5% CO₂, and then the cells were fixed in methanol for 15 min and stained with 0.05% crystal violet in PBS for 15 min. Cells on the upper sides of the filters were removed with cotton-tipped swabs, and the filters were washed with PBS. Cells on the undersides of the filters were examined and counted under a microscope.

Transfection

HCT-116 cells were transiently transfected by 0.8 µg dominant-negative mutants of ERK (DN-ERK), p38 (DN-p38), and Akt (DN-AKT), pcDNA3 empty vectors, HO-1 siRNA, or control siRNA with Lipofectamine 2000 (Invitrogen) for 24 h. Plasmid DNA and LF2000 were premixed in OPTIMedium (Invitrogen Life Technologies) for 20 min and then applied to the cells. An equal volume of medium containing 20% FBS was added 6 h later. After transfection for 24 h, LF2000-containing medium was replaced with fresh, serum-free medium and treated with BDNF for another 24 h. Transfection efficiency was determined by transfection with the plasmid with pEGFP-N1 (Clontech), which utilizes a strong cytomegalovirus (CMV)-immediate early promoter to code for an enhanced green fluorescent protein (GFP) (Supplementary Figure 1, see section on supplementary data given at the end of this article).

Quantitative real-time-PCR

Total RNA was extracted from cells using a Trizol reagent (Invitrogen) and the protocol of the PCR was performed as described in our previous reports (Chuang et al. 2014, Tsai et al. 2015). Briefly, the RT reaction was performed using a RT kit (Promega) and amplified using the following oligonucleotide primers: HO-1 forward: 5'-CACGCCCATACCCC-GCTACCT-3', reverse: 5'-TCTGTACCCCTTGTTTGC-3'; VEGF forward: 5'-CTACCCTACACATGCCAGT-3', reverse: 5'-GCATGCTGCTGCATGAGA-3'; GAPDH forward: 5'-AGGCTGCTTTAATTCTGAGG-3', reverse: 5'-GCCACTTGATTTGGAGAGGA-3'. Quantitative real-time PCR using SYBR Green Master Mix was performed by a StepOne Plus System (Applied Biosystems). The threshold was set above the non-template control background and within the linear phase of target gene amplification for calculating the cycle number at which the transcript was detected.

Western blot analysis

The whole-cell lysis extracts were prepared as described in our previous reports (Huang et al. 2013, Tsai et al. 2014). In brief, cells were lysed with a lysis buffer on ice. The supernatants were collected by centrifugation at 13 000 g for 30 min and stored at −20 °C. Protein samples were separated by SDS-PAGE and transferred to polyvinylidene-nitrofluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% nonfat milk and probed overnight with primary antibody at 4 °C. After
undergoing PBS washes, the membranes were incubated with secondary antibody. The blots were visualized by ECL using Kodak X-OMAT LS film (Eastman Kodak). The blots were subsequently stripped by incubation in stripping buffer and probed by a loading control antibody.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism 4.01 Software (GraphPad Software, Inc., San Diego, CA, USA). Values are presented as means ± S.E.M. Statistical analysis of the difference between two samples was performed using Student’s t-test.

**Results**

**BDNF induces the migration activity of human colon cancer cells**

BDNF is significantly elevated in poorly differentiated colon cancer as compared with well-differentiated colon tumors and normal tissue (Yang et al. 2013). To confirm the relationship between the BDNF expression and cell motility in colon cancer, cell migration was examined using the transwell assay. As shown in Fig. 1A, BDNF added to the medium enhanced migration activity in both HCT-116 cells (Fig. 1A) and SW480 cells (Fig. 1B) in a concentration-dependent manner. In order to investigate the role of the BDNF receptor in BDNF-mediated colon cancer cell migration, TrkB inhibitor K252a was used to examine the BDNF-induced colon cancer cell migration. Treatment of HCT-116 cells with K252a markedly inhibited BDNF-induced cell migration in colon cancer cells (Fig. 1C). Furthermore, BDNF also increased migration activity in both HCT-116 cells (Fig. 1D, upper panel) and SW480 cells (Fig. 1D, lower panel).

**BDNF-directed colon cancer cell migration involves HO-1 expression**

It has been reported that HO-1 expression is implicated in cell migration and tumor progression (Jozkowicz et al. 2007).

**Figure 1**

BDNF induces the migration activity of human colon cancer cells. HCT-116 (A) and SW480 (B) human colon cancer cells were treated with various concentrations of BDNF (25, 50, or 100 ng/ml). Transmigration activities were measured by transwell assay after BDNF treatment for 24 h. Results are expressed as the means ± S.E.M of three independent experiments. The migrated cells were visualized by phase-contrast imaging. (C) HCT-116 cells were incubated with TrkB inhibitor K252a for 30 min and were then stimulated with BDNF for 24 h. Transmigratory activity was measured by transwell assay. Results are expressed as the means ± S.E.M of three independent experiments. *P<0.05 as compared with the control group; #P<0.05 as compared with the BDNF treatment group. (D) Cells were seeded on the migration inserts for 24 h and treated with various concentrations of BDNF (25, 50, or 100 ng/ml) for another 24 h. The migrated cells were determined by transmigration assay and visualized by phase-contrast imaging.
BDNF increases HO-1 expression in human colon cancer cells. Cells were stimulated with BDNF at various concentrations (25, 50, or 100 ng/ml) for 24 h (A) or with a concentration of 100 ng/ml for the indicated time periods (B; 4, 8, or 24 h). After cell lysate extracts were collected, HO-1 protein levels were determined by western blot analysis. (C) Cells were treated with BDNF (100 ng/ml) and HO-1 mRNA expression was analyzed by RT-PCR. Results are expressed as the means ± S.E.M of three independent experiments.

Figure 3
BDNF-directed human colon cancer cell migration involves HO-1 expression. (A) Cells were treated with CoPP (0.3 or 1 μM) for 24 h. HO-1 protein levels were determined by western blot analysis (left panel), and migration activities were examined by transwell assay (right panel). Results are expressed as the means ± S.E.M of three independent experiments. *P<0.05 as compared with the control group. (B) ZnPP (B) or SnPP (C) for 30 min and were then stimulated with BDNF for 24 h. Transmigratory activity was measured by transwell assay. Results are expressed as the means ± S.E.M of three independent experiments. *P<0.05 as compared with the control group; *P<0.05 as compared with the BDNF treatment group.

The results described in our previous report also indicated that HO-1 participates in glioma cell migration (Lu et al. 2012). As shown in Fig. 2A and B, BDNF increased HO-1 protein expression in concentration- and time-dependent manners. Treatment with BDNF also increased HO-1 mRNA expression, which was determined by quantitative real-time PCR (Fig. 2C). Moreover, the transfection of cells with HO-1 siRNA for 24 h inhibited BDNF-induced colon cancer cell migration (Fig. 2D). These results indicate that BDNF induces colon cancer cell migration through HO-1 upregulation. Treatment with CoPP, an HO-1 activator, also increased colon cancer cell migration, which further supports the hypothesis that HO-1 is involved in BDNF-directed cell migration in colon cancer cells (Fig. 3A).

Furthermore, the HO-1 inhibitors ZnPP and SnPP were used to confirm the BDNF-induced cell migration. Treatment of cells with ZnPP or SnPP markedly inhibited BDNF-induced cell migration in HCT116 cells (Fig. 3B and C).

BDNF-directed colon cancer cell migration involves VEGF expression
Angiogenesis has been found to play an important role in the growth and migratory potential of colon cancer (Bagnasco et al. 2012). In the present study, we further examined whether BDNF regulates expression of the angiogenic factor VEGF in HCT-116 cells. As shown in Fig. 4A and B, BDNF increased VEGF protein expression in concentration- and time-dependent manners. In addition, BDNF increased VEGF mRNA expression in HCT-116 cells (Fig. 4C). We previously demonstrated that transfection with VEGF-luciferase reporter increases
BDNF increases VEGF expression in human colon cancer. HCT-116 cells were stimulated with BDNF at various concentrations (25, 50, or 100 ng/ml) for the 24 h (A) or with a concentration of 100 ng/ml for the indicated time periods (B; 4, 8, or 24 h). After cell lysate extracts were collected, VEGF protein levels were determined by western blot analysis. (C) Cells were treated with BDNF (100 ng/ml), and VEGF mRNA expression was analyzed by RT-PCR. Results are expressed as the means ± S.E.M. of three independent experiments. *P < 0.05 as compared with the control group. (D) Cells were transfected with the VEGF luciferase expression plasmid for 24 h. The VEGF luciferase activity was assayed after BDNF stimulation for another 8 or 24 h. Results are expressed as the means ± S.E.M. of three independent experiments. *P < 0.05 as compared with the control group; #P < 0.05 as compared with the control IgG plus BDNF treatment group.

Figure 5
The involvement of VEGF in BDNF-induced HO-1 expression in human colon cancer cells. (A) Cells were transfected with the HO-1 luciferase expression plasmid for 24 h and then incubated with VEGF inhibitor for 30 min. (B) Cells were transfected with the VEGF luciferase expression plasmid for 24 h and then incubated with ZnPP IX for 30 min. The luciferase activity was assayed after BDNF stimulation for another 24 h. Results are expressed as the means ± S.E.M. of three independent experiments. *P < 0.05 as compared with the control group; #P < 0.05 as compared with the BDNF treatment group. (C) Cells were transfected with control or HO-1 siRNA for 24 h and then were stimulated with BDNF (100 ng/ml) for another 24 h. After cell lysate extracts were collected, HO-1 and VEGF protein levels were determined by western blot analysis.

ERK, p38, and Akt signaling pathways are involved in BDNF-mediated VEGF/HO-1 upregulation and cell migration in colon cancer cells

Results of previous studies have indicated that BDNF activates MAP kinase and Akt signaling pathways (Chen et al. 2013, Revest et al. 2014). Therefore, we examined the role of Akt and MAP kinase in HO-1 activation. Our results indicated that the stimulation of cells with BDNF increased the phosphorylation of ERK, p38, and Akt but not JNK (Fig. 6A). Treatment of cells with PD98059, SB203580, or LY294002 effectively reduced the enhancement of BDNF-induced colon cancer migration (Fig. 6B).
Endocrine-Related Cancer activation and promotes colon cancer motility. and HO-1 expression through ERK, p38, and Akt and D). These results indicate that BDNF increases VEGF distant metastases (Coppede that rate decreases to less than 10% for patients with approximately 90% for early colon cancer patients, but of death from the disease. The 5-year survival rate is Surgery is the primary form of treatment for colon cancer, cell migration have also been found in colon cancer development (Shin et al. 2012, Li et al. 2014).

VEGF seems to be one of the most important mediators of tumoral angiogenesis. Results of a previous study indicated that patients with high-expression variants of VEGF were more likely to develop tumor recurrence (Lurje et al. 2008). Colon cancer patients with VEGF-positive tumors have shown increased tumor recurrence and a resistance to adjuvant chemotherapy (Cascini et al. 2001). VEGF-directed therapy improves survival for those with metastatic colon cancer (Logan-Collins et al. 2008). Interestingly, VEGF also induces HO-1 expression, which contributes to cytoprotection in acute lung injury (Siner et al. 2007). Results of a previous study also indicated that VEGF mediates the autocrine induction of HO-1 in macrophage (Weis et al. 2009). Importantly, BDNF has been reported to be a potent angiogenic factor that facilitates tumor growth by promoting VEGF expression and angiogenesis in hepatocellular carcinoma (Lam et al. 2011). Recently, we also reported that VEGF–VEGF receptor interaction increases cell motility in human colon cancer (Huang et al. 2014b). The present results support results described in previous reports that VEGF is not only secreted from colon cancer in response to BDNF, but it also causes autocrine–paracrine signaling to further induce HO-1. Therefore, targeting VEGF and its signaling pathway may become a valuable diagnosis predictor for colon cancer.

Heme oxygenase is a rate-limiting enzyme that catalyzes the conversion of heme to carbon monoxide, biliverdin, and iron (Motterlini & Foresti 2014). HO-1 is a phase II enzyme that is up-regulated in conditions of

**Discussion**

Surgery is the primary form of treatment for colon cancer, and it cures approximately 50% of patients. Recurrence following surgery is a major problem and the leading cause of death from the disease. The 5-year survival rate is approximately 90% for early colon cancer patients, but that rate decreases to less than 10% for patients with distant metastases (Coppede et al. 2014). Results of previous studies have indicated that the increased migration and invasion abilities of tumor cells are parallel to EMTs and account for the first step in the invasion of adjacent tissues by tumors and metastases in colon cancer (Hur et al. 2013). Deregulated expression of the proteins that control the cell–extracellular matrix interaction and

In addition, treatment with a specific Akt inhibitor also antagonized the enhancement of BDNF-induced cell motility (Fig. 6B). Next, we further examined whether ERK, p38/MAP kinase, and Akt signaling pathways are involved in BDNF-mediated VEGF/HO-1 upregulation in colon cancer cells. As shown in Fig. 6C and D, treatment with PD98059, SB203580, or Akt inhibitor (Akti) for 30 min and were then stimulated with BDNF for 24 h. Transmigratory activity was measured by transwell assay after BDNF treatment for 24 h. Results are expressed as the means ± s.e.m. of at least three independent experiments. *P < 0.05 as compared with the control group; **P < 0.05 as compared with the BDNF treatment group. (C) Cells were transfected with the luciferase plasmid for 24 h and then treated with PD98059, SB203580, or LY294002 for 30 min, or they were co-transfected with luciferase plasmid and ERK-DN, p38-DN, or Akt-DN for 24 h. HO-1- (C) and VEGF- (D) luciferase activity were determined. After incubation with BDNF for another 24 h, HO-1 luciferase activity was measured and the results were normalized to the β-galactosidase activity. The results are expressed as the means ± s.e.m. from three independent experiments. *P < 0.05 as compared with the control group; **P < 0.05 as compared with the BDNF treatment group.

**Figure 6**

The involvement of MAP kinase/Akt in BDNF-induced HO-1 expression in human colon cancer cells. (A) Cells were incubated with BDNF for the indicated time periods. The phosphorylation of ERK, p38, Akt, and JNK were determined by western blot analysis. (B) Cells were incubated with PD98059, SB203580, LY294002, or Akt inhibitor (Akti) for 30 min and were then stimulated with BDNF for 24 h. Transmigratory activity was measured by transwell assay after BDNF treatment for 24 h. Results are expressed as the means ± s.e.m. of at least three independent experiments. *P < 0.05 as compared with the control group; **P < 0.05 as compared with the BDNF treatment group.
BDNF has been shown to activate the MAP kinase pathways in the regulation of cell migration and differentiation (Petridis & El Maarouf 2011). It is known that the MAP kinase and Akt pathways play important roles in colon cancer cell invasion and migration (Tremblay et al. 2006, Huynh et al. 2010, Slattery et al. 2012). The activation of Akt signaling promotes EMT and tumor growth in colon cancer cells (Suman et al. 2014). In the present study, we showed that BDNF induced cell motility and upregulated VEGF expression via the induction of HO-1. Moreover, BDNF-induced cell motility was also modulated by the ERK, p38, and Akt pathways in colon cancer cells. Furthermore, inhibition of the ERK, p38, and Akt pathways effectively antagonized the BDNF-induced HO-1 and VEGF activity. These results indicate that BDNF activates VEGF expression and HO-1 induction via the ERK, p38, and Akt signaling pathways, which thereby contributes to cell motility in human colon cancer.

Hence, the inhibition of migratory pathways and the molecular mechanism of angiogenesis is an attractive target for colon cancer treatment. There is an urgent need to understand the cellular and molecular factors that promote cancer cell motility. A better understanding of the molecular mechanisms of colon cancer progression may help us find prognostic biomarkers and to develop biological agents for treating colon cancer.
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