Research Article

Antioxidant Peptide Derived from *Spirulina maxima* Suppresses HIF1 α -Induced Invasive Migration of HT1080 Fibrosarcoma Cells

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Hypoxia causes the malignant progression of tumor cells; hence, it has been considered a central issue that must be addressed for effective cancer therapy. The initiation of tumor metastasis requires invasive cell migration. Here, we show that an antioxidant peptide derived from *Spirulina maxima* suppresses hypoxia-induced invasive migration of HT1080 human fibrosarcoma cells. HT1080 cells treated with a hypoxia-inducing agent, CoCl₂, exhibited an increase in invasive migration and intracellular reactive oxygen species (ROS), which is associated with an increase in the expression of hypoxia-induced factor 1 α (HIF1 α) accompanied by the activation of PI3K/Akt and ERK1/2. The inhibition of PI3K/Akt and ERK1/2 with specific inhibitors diminished the CoCl₂-induced increase in HIF1 α expression and invasive cell migration. Moreover, CoCl₂-induced HIF1 α expression was associated with an increase in the expression of molecules downstream of β -integrin, such as N-cadherin, vimentin, and β -catenin. Therefore, the *S. maxima* peptide effectively attenuated the CoCl₂-induced ROS generation and downregulated the HIF1 α signaling pathway involving PI3K/Akt, ERK1/2, and β -integrin in cells. These results suggest that the *S. maxima* antioxidant peptide downregulates the HIF1 α signaling pathway necessary for hypoxia-induced invasive migration of HT1080 cells by attenuating intracellular ROS. *S. maxima* peptide may be an effective constituent in antitumor progression products.

1. Introduction

The rapid growth and proliferation of tumor cells result in a dramatic surge in oxygen demand. Tumor hypoxia caused by inadequate oxygen supply is strongly associated with tumor propagation, malignant progression, and resistance to therapy [1, 2]. Therefore, tumor hypoxia is an important factor in tumor biochemistry and an important challenge in tumor treatment. Accumulating evidence indicates that the effect of hypoxia on the malignant progression of tumor cells is mediated by a series of hypoxia-induced cellular changes that activate anaerobic metabolism, angiogenesis, and metastasis and enable tumor cells to survive or escape their oxygen-deficient environment [1, 3]. The transcription factor hypoxia-inducible factor 1 (HIF1), containing HIF1 α and HIF1 β subunits, has been known to be a key regulator

in tumor cell adaptation to hypoxic environments. In particular, HIF1 α regulates the expression of a number of genes affecting the metastatic progression of tumor cells [4–6]. Tumor metastasis is a complex multistep process by which tumor cells disseminate from the primary site, penetrate into lymphatic and blood vessels, and spread to other sites in the body. HIF1 α expression has been implicated in the increased invasive migration of tumor cells during the initiation of metastasis [2, 7].

Reactive oxygen species (ROS) have been recently identified as key mediators involved in tumor propagation and malignant progression. ROS activate downstream PI3K/Akt, ERK1/2, and β -integrin pathways that regulate HIF1 α expression [8–10]. ROS-induced upregulation of HIF1 α also causes the invasive migration of tumor cells through the regulation of target genes such as N-cadherin, vimentin, and β -catenin

[2, 7]. Therefore, there has been a marked increase in the study of the role of ROS and antioxidants in the prevention of tumor progression.

Peptides purified from protein hydrolysates have received much attention because of their anticancer, anti-inflammation, and antioxidant activities [11, 12]. We recently reported that an antioxidant peptide identified from the enzymatic hydrolysates of *Spirulina maxima* is effective against FcεRI-mediated allergic reactions in mast cells [13]. In the present study, we examined the protective effects of this peptide against the invasive migration of tumor cells *in vitro*.

2. Materials and Methods

2.1. Cell Culture and Reagents. HT1080 human fibrosarcoma cells, obtained from the American Type Culture Collection (ATCC), were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin, in 5% CO₂ at 37°C. For the cell-culture experiments, cells were passaged at least 3 times and detached with trypsin-EDTA. Matrigel Invasion Chambers were purchased from BD Biosciences (USA). Antibodies against HIF1α, Akt, ERK1/2, N-cadherin, vimentin, β-catenin, and actin were obtained from BD Biosciences (USA), Santa Cruz Biotechnology (USA), Cell Signaling Technology (USA), and Sigma-Aldrich (USA), respectively. DCFH-DA was purchased from Molecular Probes. Chemicals and reagents were purchased from Sigma-Aldrich, unless stated otherwise.

2.2. Preparation of the *Spirulina maxima* Peptide. The *S. maxima* peptide was prepared as reported by Vo et al. [13]. The purity of the peptide was >98% according to RP-HPLC assessment and N-terminal sequence analysis. The amino acid sequence of the final purified peptide was determined to be LDAVNR by electrospray ionization mass spectrometry (ESI/MS).

2.3. Cell Viability (MTT) Assay. The cytotoxicity of CoCl₂ and/or peptide was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] formazan assay. HT1080 cells were seeded in 96-well plates at a density of 1×10^3 cells/well in DMEM containing 10% fetal bovine serum. Twenty-four hours after seeding, the medium was changed to DMEM containing 0.1% bovine serum albumin (DMEM-BSA) and the cells were incubated with 100 μM CoCl₂ with or without 100 μM peptide for 24 h. Thereafter, the medium was carefully removed and 160 μL of MTT (0.5 mg/mL final concentration) solution was added to each well prior to incubation for an additional 4 h at 37°C in 5% CO₂. The medium was aspirated without the formazan crystals and 1 mL of DMSO was added to each well. The absorbance was measured on a microplate reader (iMark, Bio-Rad) at 540 nm.

2.4. Invasive Cell Migration Assay. For the invasion assay, the lower surface of the porous membranes in the Matrigel Invasion Chambers (BD Biosciences, USA) was coated with

fibronectin (25 μg/mL) at room temperature for 1 h and washed 3 times in DMEM-BSA. DMEM-BSA was added to the lower compartment of the chamber. Cells were starved in DMEM-BSA overnight and treated with 100 μM CoCl₂ or 100 μM peptide as described above, trypsinized, and collected. Thereafter, 200 μL of each cell suspension (2×10^5 cells/well in DMEM-BSA) was added to the upper compartment of the chamber and incubated at 37°C in a humidified atmosphere with 5% CO₂ for 24 h. Cells on the upper surface of the membrane were removed, and cells that had migrated to the lower surface of the membrane were fixed with 3.7% formaldehyde in phosphate buffered saline (PBS), stained with crystal violet (0.4% dissolved in 10% ethanol) for 15 min, washed twice with PBS, and counted under a phase-contrast microscope with a 10x objective lens. Cells in 9 randomly selected fields from triplicate chambers were counted in each experiment.

2.5. Measurement of ROS. Dichlorofluorescein diacetate (DCF-DA) was used to evaluate the generation of ROS by oxidative stress. HT1080 cells (4×10^4 cells/well) in 24-well plates were first incubated with 100 μM CoCl₂ or 100 μM peptide for 24 h. The cells were then washed with PBS and incubated with 10 μM DCFH-DA for 30 min at room temperature. Fluorescence was measured by using a fluorescence plate reader. Furthermore, cellular ROS levels were determined by dihydroethidium (DHE) (Sigma-Aldrich) staining and by using the Muse Oxidative Stress Kit (Millipore) according to the manufacturer's instructions.

2.6. Western Blotting. After proper treatment, cells were washed 2 times with PBS, harvested, and solubilized in 2x sodium dodecyl sulfate (SDS) protein sample buffer containing 100 mM Tris-HCl (pH 6.8), 200 mM DTT, 4% SDS, 0.4% bromophenol blue, and 20% glycerol. Equal amounts of protein samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE). The resolved proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp.). The membranes were blocked by incubation with 1% bovine serum albumin (BSA) in TBS-T (10 mM Tris-HCl, 150 mM NaCl [pH 7.5], with 0.1% Tween-20) at room temperature for 1 h and further incubated with the specific primary antibody for 1 h. The membranes were washed 3 times with TBS-T and incubated for 30 min with the appropriate secondary antibody conjugated to alkaline phosphatase (AP). Proteins were detected by colorimetric reactions using the respective BCIP/NBT substrates.

3. Results

3.1. *S. maxima* Peptide Decreases CoCl₂-Induced Invasive Migration of HT1080 Cells. We first examined whether the peptide purified from *S. maxima* affects the invasive migration of highly metastatic HT1080 fibrosarcoma cells. HT1080 cells were precultured in the presence or absence of *S. maxima* peptide for 24 h, transferred onto Matrigel-coated transwell membranes, and further incubated for 16 h under preculture conditions. Cells that had migrated to the lower

FIGURE 1: *S. maxima* peptide decreases CoCl₂-induced invasive migration of HT1080 cells. HT1080 cells were serum starved in DMEM containing 0.1% bovine serum albumin (DMEM-BSA) for 24 h and precultured in the presence or absence of 100 μM CoCl₂ and/or various concentrations of *S. maxima* peptide for 24 h, transferred onto Matrigel-coated transwell membranes, and further incubated for 16 h under preculture conditions. Migrated cells on the lower surface of the membrane were fixed, stained, and counted. Cells in 9 randomly selected fields were counted in each experiment. Results of at least 3 independent experiments were averaged. All data are presented as mean ± S.D. **P* < 0.05 compared with blank; †*P* < 0.05 compared with CoCl₂ control.

surface of the membrane were fixed, stained, and counted under a phase-contrast microscope. The results demonstrated that treatment with the *S. maxima* peptide inhibited the invasive migration of HT1080 cells (Figure 1(a)). After treatment with the peptide, invasive migration was decreased to 97.4%, 84.7%, and 78.3% (*P* < 0.05) at 25, 50, and 100 μM of peptide, respectively. Interestingly, the inhibitory effect of the *S. maxima* peptide on the invasive migration of HT1080 cells was more dramatic under CoCl₂-induced hypoxic condition (Figure 1(b)). HT1080 cells incubated with 100 μM CoCl₂ demonstrated an approximately 1.7-fold increase in invasive migration compared to that of blank which were not incubated with CoCl₂. Additional treatment with the *S. maxima* peptide significantly attenuated CoCl₂-induced increase in the invasive migration of cells. Compared to control cells without peptide treatment, treatment with 25, 50, and 100 μM of the peptide reduced the invasive migration of the cells to 85.2%, 66.2%, and 54.7%, respectively. Since treatment with <100 μM of the *S. maxima* peptide did not affect cell viability (Figure 2), these results suggest that the *S. maxima* peptide regulates intracellular signaling involved in the hypoxic invasive migration of HT1080 cells.

3.2. *S. maxima* Peptide Effectively Decreases CoCl₂-Induced ROS Generation in HT1080 Cells. Since many studies have shown that elevated ROS generation under hypoxic conditions is associated with tumor progression and metastasis, and that the *S. maxima* peptide decreases ROS production in FcεRI-mediated mast cell activation [13], we attempted to examine whether the *S. maxima* peptide regulates CoCl₂-induced ROS generation in HT1080 cells. The fluorescent probe DCFH-DA was used to measure the effect of the *S. maxima* peptide on the intracellular ROS level in HT1080 cells. As shown in Figure 3(a), CoCl₂ treatment increased the ROS level in HT1080 cells, whereas the *S. maxima*

FIGURE 2: Effect of the *S. maxima* peptide and CoCl₂ on the viability of HT1080 cells. HT1080 cells were incubated with 100 μM CoCl₂ and various concentrations of *S. maxima* peptide for 48 h and assessed using the MTT assay. Results of 3 independent experiments were averaged. All data are presented as mean ± S.D.

peptide effectively attenuated the CoCl₂-induced increase in the ROS levels. Treatment with 100 μM CoCl₂ increased the intracellular ROS level to approximately 2.57-fold compared to that in blank without CoCl₂ stimulation. In contrast, the addition of 100 μM *S. maxima* peptide attenuated the CoCl₂-induced increase in ROS levels to similar level to that in control cells without CoCl₂ stimulation (Figure 3(a)). Similarly, microscopic fluorescence image and flow cytometry assays showed that the *S. maxima* peptide is effective in the suppression of CoCl₂-induced ROS generation (Figures 3(b) and 3(c)). Figure 3(c) shows that the addition of 100 μM *S. maxima* peptide decreased CoCl₂-induced ROS generation (67.42%) in HT1080 cells to approximately 37.14%. Therefore, these data suggest that the *S. maxima* peptide may regulate the hypoxia-induced increase in intracellular ROS levels.

FIGURE 3: *S. maxima* peptide attenuates CoCl₂-induced ROS level in HT1080 cells. (a) HT1080 cells (4×10^4 cells/well) were incubated in the presence or absence of 100 μ M CoCl₂ and 100 μ M peptide for 24 h. Cellular ROS levels were assessed by DCFH-DA. All data are presented as mean $\pm$ S.D. * $P < 0.05$ compared with blank; † $P < 0.05$ compared with CoCl₂ control. (b) Representative images of HT1080 cells stained with DCF-DA. Intracellular ROS is observed with fluorescence microscopy (ZEISS, MIC00266). Bar, 10 μ m. (c) Cellular ROS levels were determined by dihydroethidium (DHE) staining and flow cytometry assay. Representatives of at least 3 independent experiments are shown in the panel.

FIGURE 4: *S. maxima* peptide suppresses the HIF1 α signaling pathway necessary for CoCl₂-induced invasive migration in HT1080 cells. (a) HT1080 cells were incubated with 100 μ M CoCl₂ and/or 100 μ M peptide for 24 h in the presence or absence of PI3K inhibitor, 10 μ M LY294002 or ERK inhibitor, and 10 μ M PD98059. Cell lysates were prepared, and the expression level of HIF1 α and phosphorylation levels of Akt and ERK1/2 were examined by western blot analysis. Representatives of 3 independent experiments are shown. (b) Cells were prepared as in (a). Invasive migration of HT1080 cells was examined. All data are presented as mean $\pm$ S.D. * $P < 0.05$ compared with blank without CoCl₂ stimulation; † $P < 0.05$ compared with CoCl₂ control. (c) HT1080 cells were incubated with or without 100 μ M CoCl₂ and 100 μ M peptide for 24 h. Cell lysates were analyzed for HIF1 α , β -integrin, N-cadherin, vimentin, β -catenin, and β -actin.

3.3. *S. maxima* Peptide Downregulates the HIF1 α Signaling Pathway Necessary for CoCl₂-Induced Invasive Migration of HT1080 Cells. Hypoxia-induced cellular ROS induces the expression and activation of transcription factor hypoxia-inducible factor 1 α (HIF1 α), which leads to aggressive cellular changes that are associated with tumor cell invasion through the regulation of target genes [2, 7]. Since PI3K/Akt and ERK1/2, upstream molecular regulators of HIF1 α , have been reported to be responsible for invasive migration in response to oxidative stress during tumorigenesis [14, 15], we first examined whether these proteins are further involved in the CoCl₂-induced hypoxic condition of HT1080 cells. HT1080 cells were incubated with 100 μ M CoCl₂ for 24 h in the presence or absence of PI3K inhibitor, LY294002 or ERK inhibitor, and PD98059. Thereafter, the expression level of HIF1 α , phosphorylation levels of Akt and ERK1/2, and invasive migration of HT1080 cells were examined (Figures 4(a) and 4(b)). CoCl₂ treatment increased the phosphorylation levels of Akt and ERK1/2 as well as expression level of HIF1 α in HT1080 cells. However, LY294002 or PD98059 treatment reduced the CoCl₂-induced increase in the expression of HIF1 α and phosphorylation levels of Akt and ERK1/2 (Figure 4(a)). The CoCl₂-induced invasive migration of HT1080 cells was decreased by treatment of

these inhibitors (Figure 4(b)), indicating that PI3K/Akt and ERK1/2, which are upstream of HIF1 α , are associated with CoCl₂-induced invasive migration of HT1080 cells.

We also examined whether the *S. maxima* peptide regulates the PI3K/Akt and ERK1/2 signaling necessary for CoCl₂-induced invasive migration of HT1080 cells (Figure 4(a)). HT1080 cells were incubated with 100 μ M CoCl₂ for 24 h in the presence or absence of 100 μ M *S. maxima* peptide, and the expression and phosphorylation levels of the related proteins were examined. Treatment with the *S. maxima* peptide attenuated the CoCl₂-induced increase in the phosphorylation levels of Akt and ERK1/2 as well as expression of HIF1 α . Furthermore, CoCl₂ treatment was found to increase the expression of β -integrin, another regulator of HIF1 α , and the *S. maxima* peptide conversely attenuated CoCl₂-induced increases in the expression of β -integrin (Figure 4(c)). Since many studies have previously indicated that the ROS/HIF1 α -induced activation of β -integrin is associated with the activation of N-cadherin, vimentin, and β -catenin, the underlying mechanisms were further investigated (Figure 4(c)). The treatment of HT1080 cells with CoCl₂ increased the expression levels of N-cadherin, vimentin, and β -catenin, whereas the *S. maxima* peptide treatment conversely attenuated the CoCl₂-induced increase in the expression levels of these

proteins. Taken together, we confirmed that the antioxidant *S. maxima* peptide downregulates the HIF1 α signaling pathway necessary for hypoxia-induced invasive migration of HT1080 cells by attenuating intracellular ROS.

4. Discussion

Tumor hypoxia has been regarded as a potential therapeutic problem since it can cause a more aggressive malignant progression of tumor cells. Sustained tumor hypoxia increases the potential for invasive growth and metastatic migration and enhances the intrinsic resistance of tumors to cancer treatments [1, 2]. Therefore, an understanding of the effects of hypoxia on tumor physiology is required to counteract tumor progression. Recently, it has been accepted that the ROS-induced upregulation of HIF1 α causes the invasive migration of tumor cells through the regulation of genes such as PI3K/Akt, ERK1/2, and β -integrin/catenin [8–10]. Therefore, there has been a marked increase in the study of ROS and antioxidants for the prevention of tumor progression through the HIF1 α signaling pathway. In this study, we showed that an antioxidant peptide derived from *S. maxima* attenuates CoCl₂-induced intracellular ROS generation and downregulates the HIF1 α signaling pathway, leading to a decrease in the invasive migration of HT1080 fibrosarcoma cells.

Previous studies have reported that hypoxia-induced ROS activate downstream PI3K/Akt and ERK1/2 pathways that regulate HIF1 α . The inhibition of ROS production reduces not only the phosphorylation of Akt and ERK1/2 but also the expression of HIF1 α , which is associated with a decrease in cell migration and invasion [8–10]. The suppression of PI3K/Akt or ERK1/2 inhibits the migration of tumor cells in response to the extracellular matrix and growth factors under hypoxic conditions [16–18]. Therefore, these results suggest a dependency of ROS on the PI3K/Akt and ERK1/2 signaling pathway and HIF1 α expression in invasive tumor cell migration. Our results indicate that these HIF1 α upstream molecules are further involved in CoCl₂-induced invasive migration of HT1080 cells, and that the antioxidant *S. maxima* peptide has antitumor effects based on this molecular mechanism. In addition, the β -integrin family, which is heterodimeric receptors involved in cell-cell and cell-extracellular matrix interactions [19], has been reported to promote invasive cell migration [20]. A relationship between the metastatic potential of tumor cells during invasion and quantitative changes in β -integrin expression has been reported in several hypoxic cancers [21, 22]. The upregulation of β -integrin expression is associated with the upregulation of epithelial-mesenchymal transition marker proteins such as N-cadherin and vimentin, and the downregulation of E-cadherin which is a cell-cell adhesion molecular marker in cancer cells [14, 21]. Therefore, it has been suggested that the regulation of molecules downstream of β -integrin such as N/E-cadherin, vimentin, and β -catenin is also important for hypoxia-induced invasive migration of tumor cells. The present results clearly support this hypothesis by demonstrating the relationship between the expression of HIF1 α and N-cadherin, vimentin, and β -integrin/ β -catenin in hypoxia-induced invasive tumor cell migration. Taken together, our

results suggest that the antioxidant peptide derived from *S. maxima* may be an effective constituent in antitumor progression products based on the mechanism of tumor progression through ROS and the HIF1 α signaling pathway.

5. Conclusion

The *S. maxima* peptide attenuates the CoCl₂-induced intracellular ROS generation and downregulates the HIF1 α signaling pathway involving PI3K/Akt, ERK1/2, and β -integrin in HT1080 cells, which results in a decrease in the CoCl₂-induced invasive migration of HT1080 fibrosarcoma cells.

Conflict of Interests

The authors declare no conflict of interests.

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