Effects of proton beam irradiation on mitochondrial biogenesis in a human colorectal adenocarcinoma cell line

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Abstract. Proton beam therapy has recently been used to improve local control of tumor growth and reduce side-effects by decreasing the global dose to normal tissue. However, the regulatory mechanisms underlying the physiological role of proton beam radiation are not well understood, and many studies are still being conducted regarding these mechanisms. To determine the effects of proton beams on mitochondrial biogenesis, we investigated: mitochondrial DNA (mtDNA) mass; the gene expression of mitochondrial transcription factors, functional regulators, and dynamic-related regulators; and the phosphorylation of the signaling molecules that participate in mitochondrial biogenesis. Both the mtDNA/nuclear DNA (nDNA) ratio and the mitochondria staining assays showed that proton beam irradiation increases mitochondrial biogenesis in 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced aggressive HT-29 cells. Simultaneously, proton beam irradiation increases the gene expression of the mitochondrial transcription factors PGC-1α, NRF1, ERRα, and mtTFA, the dynamic regulators DRP1, OPA1, TIMM44, and TOM40, and the functional regulators CytC, ATP5B and CPT1-α. Furthermore, proton beam irradiation increases the phosphorylation of AMPK, an important molecule involved in mitochondrial biogenesis that is an energy sensor and is regulated by the AMP/ATP ratio. Based on these findings, we suggest that proton beam irradiation inhibits metastatic potential by increasing mitochondrial biogenesis and function in TPA-induced aggressive HT-29 cells.

Introduction

Colorectal cancer (CRC) is steadily increasing worldwide, but has a particularly high incidence in developed countries. Approximately 20% of CRC patients already have metastases at diagnosis (1). It is well known that metastasis spreads cancer to other organs such as the liver, lungs and lymph nodes, and it accounts for over 90% of cancer-related mortality (2).

Mitochondria play an important role in the production of ATP via oxidative phosphorylation (OXPHOS). Mitochondria are also involved in biosynthetic metabolic processes involving cholesterol, heme, lipids and nucleotides. Moreover, mitochondria are considered the main sources of reactive oxygen species and nitrogen species (3). Mitochondrial biogenesis is a complex process that occurs by the cooperation of numerous genes between nuclear and mitochondrial genomes. It is regulated by a complex transcriptional network of key factors including peroxisome proliferator-activated receptor co-activator 1 alpha (PGC-1α), the nuclear respiratory factors (NRFs), estrogen receptor-related receptor alpha (ERRα) and mitochondrial transcription factor A (mtTFA) (4).

Research has shown an association between mitochondrial biogenesis and metastasis. Cells with depleted mitochondrial DNA (mtDNA) exhibit increased invasiveness through the upregulation of cathepsin L expression (5). Depletion of mtDNA also promotes invasion by modulating the expression of matrix metalloproteinases, tissue inhibitors of the MMP family and other invasion-related genes (6). However, increased mitochondrial biogenesis is induced by treatment with bezafibrate, a pan agonist of peroxisome proliferator-activated receptors (PPARs), in cancer cells such as 143B, HeLa and MDA-MB-231. Bezafibrate treatment reduces cell growth, glucose utilization and invasion in these cells (7). When wild-type KISS1, a metastasis suppressor, is upregulated, aerobic glycolysis decreases and OXPHOS predominates. KISS1-expressing cells show that increased mitochondrial biogenesis is accompanied by increased mitochondrial gene expression and higher expression of PGC-1α, a key co-activator that regulates mitochondrial mass and metabolism (8).

Proton beam therapy (PBT), an alternative to gamma or X-ray irradiation therapy, has produced promising clinical results worldwide (9). In particular, PBT has recently been used to treat hepatocellular carcinoma (10), non-small cell lung (11), prostate (12) and head and neck tumors (13). Some in vitro studies have shown that PBT inhibits metastatic characteris-
tics such as the increase of adhesion and migration (14,15). In previous studies, we also found that proton beam irradiation inhibits metastatic potentials such as migration, invasion, and the expression of MMP-2 and MMP-9 in HT-29 cells (16,17). Furthermore, proton beam irradiation inhibits gene expression and the activities of molecules involved in integrin trafficking and integrin-mediated signaling, which are essential processes in tumor progression (18). However, the contribution of abnormal mitochondrial biogenesis to colon cancer metastasis is unknown. In the present study, we investigated the effects of proton beam irradiation on mitochondrial biogenesis by determining: mtDNA mass; the expression of mitochondrial-specific transcription factors, dynamic regulators, and functional molecules; and the activities of signaling molecules in 12-O-tetradecanoyl phorbol-13-acetate (TPA)-induced aggressive HT-29 cells.

Materials and methods

Materials. The following items were purchased from the stated commercial sources: TPA (P1585) and 4,6-diamidino-2-phenylindole (DAPI, D9542) were from Sigma-Aldrich Co. (St. Louis, MO, USA); rabbit anti-human phospho AMPK (#2535), mouse anti-human AMPK (#2793), and rabbit anti-human phospho SIRT1 (#2314) antibodies were from Cell Signaling Technology (Danvers, MA, USA); rabbit anti-human SIRT1 (sc-15404), rabbit anti-human PGC-1α (sc-13067), mouse anti-human β-actin (sc-47778), goat anti-mouse IgG-horseradish peroxidase (HRP) (sc-2005), and goat anti-rabbit IgG-HRP (sc-2004) secondary antibodies were from Santa Cruz Biotechnology Inc., (Santa Cruz, CA, USA); ECL Plus Western Blotting Substrate (34580) was from Pierce Biotechnology (Rockford, IL, USA); TRIzol (15596026) and MitoTracker Red CMXRos (M7512) were from Invitrogen-Life Technologies (Carlsbad, CA, USA); SYBR-Green Master Mix (04913850001) was from Roche USA; ECL Plus Western Blotting Substrate (34580) was from Pierce Biotechnology (Rockford, IL, USA); TRIzol (15596026) and MitoTracker Red CMXRos (M7512) were from Invitrogen-Life Technologies (Carlsbad, CA, USA); the FastStart Universal SYBR-Green Master Mix (04913850001) was from Roche Applied Science (Mannheim, Germany); and the Phosphatase Inhibitor Cocktail (P3200) and Protease Inhibitor Cocktail (P3100) solutions were from GenDEPOT (Barker, TX, USA).

Cell culture. As previously described in detail (18), the HT-29 cell line was purchased from the Korean Cell Line Bank (KCLB no. 30038, Seoul, Republic of Korea). Cells were maintained in 5% CO2 at 37°C in Roswell Park Memorial Institute (RPMI-1640) medium including 10% fetal bovine serum (FBS; SH30084.03; HyClone Laboratories-GE Healthcare Life Science, Victoria, Australia) and 100 U/ml penicillin-streptomycin (15140122; Invitrogen). For induction of metastatic capacity, cells were incubated with 150 nM TPA for 1 h and then RPMI-1640 medium was added before proton beam irradiation.

Proton beam irradiation. According to previous studies (19), proton beam irradiation was performed with a 100-MeV proton beam using the 100 MeV linac equipment (Scanditronix, Uppsala, Sweden) at the Korea Multi-purpose Accelerator Complex (KOMAC, Gyeongju, Republic of Korea). Briefly, cells in a 12.5-cm² flask filled with RPMI-1640 medium were placed on a beam stage and irradiated (1, 4 and 8 Gy) at the center of the Bragg peaks modulated to 6-cm widths. The dose during each beam irradiation was measured using radiochromic film (GAF-MD55; GAF Corp., Wayne, NJ, USA) as an in situ measuring tool.

Mitochondrial biogenesis analysis. We cultured the irradiated cells for 48 and 72 h to investigate the effect of proton beam irradiation on mitochondrial biogenesis. As previously described in detail (20), DNA from cells was extracted using a genomic DNA purification kit (A1120; Promega Corp., Madison, WI, USA), according to the manufacturer's instructions. Mitochondrial biogenesis was determined by the ratio of cytochrome c oxidase subunit I (COX I) for mtDNA to ribosomal protein large p0 (RPLP0) for nuclear DNA (nDNA), quantified by quantitative reverse transcription polymerase chain reaction (RT-qPCR). Data were normalized against the expression of RPLP0.

Mitochondrial staining and quantification. As previously described in detail (20), the irradiated cells were cultured in 8-well chamber slides (12-565-8; Thermo Fisher Scientific, Rochester, NY, USA) for 48 and 72 h. After washing with phosphate-buffered saline (PBS), the cells were incubated with RPMI-1640 medium containing 20 nM MitoTracker Red CMXRos at 37°C for 30 min. After washing with PBS, the cells were fixed with 4% paraformaldehyde for 10 min, and stained with 1 µg/ml DAPI for 10 min. Fluorescent images were obtained using a digital fluorescence microscope (Nikon Eclipse 80i; Nikon, Tokyo, Japan) at ×400 magnification. For quantification of the fluorescent signal, the cells were dissolved with a lysis buffer (0.1 M potassium phosphate, 1% Triton X-100, pH 10.0) for 10 min. Dimethyl sulfoxide (DMSO) was added to the lysates, which were then incubated for 10 min. The fluorescent signal intensity from the lysates was measured using a microplate reader (FLUOstar OPTIMA; BMG Labtech, Ortenberg, Germany) at 466 nm excitation and 540 nm emission.

RT-qPCR analysis. As previously described in detail (20), total RNA from cells was obtained using TRIzol (Invitrogen), and cDNA was produced using a PrimeScript First strand cDNA Synthesis kit (Takara Bio Inc.) according to the manufacturer's instructions. To analyze gene expression, RT-qPCR was conducted using a FastStart SYBR-Green Master mix (Roche Diagnostics) in an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Levels of target gene expression were calculated relative to the expression level of the endogenous reference gene, β-actin, using the delta cycle threshold method (Table I).

Western blot analysis. As previously described in detail (20), the irradiated cells were cultured for 48 and 72 h, and lysed in radio-immunoprecipitation assay (RIPA) buffer (89900; Thermo Fisher Scientific, Waltham, MA, USA). Total protein lysates were separated by 8% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (10 401 396; Whatman GmbH, Dassel, Germany). The membranes were incubated with 5% non-fat milk for 1 h at 25°C, and incubated with the primary antibody (1:1,000)
overnight at 4˚C. After washing with Tris-buffered saline (TBS) containing 0.1% Tween-20 for 30 min, the membranes were incubated with the anti-mouse IgG, or anti-rabbit IgG, HRP-conjugated secondary antibodies (1:3,000) for 1 h at 25˚C. After washing with TBS containing 0.1% Tween-20 for 30 min, the western blotting images were obtained with an ECL Plus Western blotting substrate (Pierce Biotechnology), using a Lumino image analyzer (LAS 4000 mini; Fujifilm, Tokyo, Japan), and quantified with ImageJ software (ver. 1.51j; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All data are shown as the mean ± SEM. The data were analyzed by the one-way analysis of variance (ANOVA). Differences between mean values were assessed using the Dunnett’s multiple comparisons test. Statistical significance was defined as P<0.05.

Results

**Proton beam irradiation stimulates mitochondrial biogenesis in TPA-induced aggressive HT-29 cells.** To determine whether proton beam irradiation regulates mitochondrial biogenesis, we analyzed the gene expression ratio of COX I and RPLP0, representing mtDNA and nDNA, respectively, in TPA-induced aggressive HT-29 cells after irradiation with a proton beam at 1, 4 or 8 Gy. Proton beam irradiation increased mitochondrial biogenesis in a dose- and time-dependent manner (Fig. 1A). At the same time, proton beam irradiation increased the expression of PGC-1α, a key transcription factor for mitochondrial biogenesis, in a dose-dependent manner (Fig. 1B). To determine whether proton beam irradiation-induced mitochondrial biogenesis is involved in mitochondrial function, we next stained mitochondria in TPA-induced aggressive HT-29 cells using MitoTracker Red CMXRos, a mitochondrial membrane potential (ΔΨm)-sensitive fluorochrome that accumulates membrane potential-dependently in live cells. Proton beam irradiation increased mitochondrial staining compared with staining in the group treated with TPA alone (Fig. 2).

**Proton beam irradiation stimulates mitochondrial biogenesis by AMPK signaling pathways in TPA-induced aggressive HT-29 cells.** To further study the mechanism by which proton beam irradiation affects mitochondrial biogenesis and function, we next investigated the phosphorylation of AMPK and SIRT1, which are upstream molecules associated with PGC-1α.
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and regulators of mitochondrial biogenesis. As shown in Fig. 3, TPA treatment decreased AMPK phosphorylation, whereas proton beam irradiation increased AMPK phosphorylation in a dose- and time-dependent manner. However, proton beam irradiation did not affect SIRT1 phosphorylation. Therefore, these results indicate that the proton beam irradiation-induced increase in mitochondrial biogenesis might be regulated by the AMPK signaling pathways.
Proton beam irradiation stimulates the expression of mitochondrial transcription factors and mitochondria functional genes in TPA-induced aggressive HT-29 cells. To further investigate the underlying regulatory mechanism associated with mitochondrial biogenesis, we investigated the mRNA expression of various genes involved in mitochondrial transcription, mitochondrial dynamics, the electron transport chain, ATP synthesis and fatty acid oxidation. As shown in Fig. 4, proton beam irradiation increased the gene expression of the transcriptional co-activators NRF1, mtTFA and ERRα in a dose- and time-dependent manner. At the same time, proton beam irradiation increased the gene expression of dynamin-related protein 1 (DRP1; a GTPase that regulates mitochondrial fission), optic atrophy 1 (OPA1; a dynamin-related GTPase, which participates in mitochondrial cristae remodeling), translocase of outer

**Figure 3.** Effects of proton beam irradiation on AMPK and SIRT1 activity in 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced aggressive HT-29 cells. (A) After irradiation at the indicated doses, western blot analysis was performed with anti-phospho AMPK, anti-AMPK, anti-phospho SIRT1 and anti-SIRT1 antibodies at the indicated times. (B) Quantitative analysis of western blot results was performed using ImageJ software. Each value represents the mean ± SEM. *P<0.05, **P<0.01 vs. the CON group.

**Figure 4.** Effect of proton beam irradiation on gene expression of mitochondrial key transcription factors in 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced aggressive HT-29 cells. After irradiation at the indicated doses, the expression levels of NRF1, mtTFA and ERRα were determined by quantitative reverse transcription polymerase chain reaction (RT-qPCR) at the indicated times. Each value represents the mean ± SEM. *P<0.05, **P<0.01 vs. the CON group.
mitochondrial membrane 40 (TOMM40), and translocase of inner mitochondrial membrane 44 (TIMM44), which play a role in mitochondrial protein import (Fig. 5). Moreover, in the context of mitochondrial function, proton beam irradiation increased the gene expression of cytochrome c (CytC), an essential component of the electron transport chain, ATP synthase subunit β (ATP5B), a subunit of mitochondrial ATP synthase, and carnitine palmitoyltransferase 1-α (CPT1-α), a mitochondrial enzyme responsible for the β-oxidation of long-chain fatty acids, in a dose- and time-dependent manner (Fig. 6). Therefore, these results indicate that proton beam irradiation is an effective stimulator of mitochondrial biogenesis and function in TPA-induced aggressive HT-29 cells.

**Discussion**

Proton beam therapy (PBT) is a new form of radiotherapy used to treat cancer. Compared with other radiotherapies such as gamma and X-ray, PBT has better dose distribution owing to its unique absorption spectrum in tissues, known as the Bragg’s peak, which allows the deposition of devastating energy at the tumor while limiting the damage to normal tissues (21). However, the biological effects and detailed mechanism of proton beam irradiation on cancer metabolism are still unclear, and further studies are needed to determine the biological role of proton beam irradiation for efficient application. In the present study, we proposed that proton beam irradiation suppresses colon cancer metastasis by stimulating mitochondrial biogenesis and inducing the expression of mitochondrial transcription factors, functional regulators, and dynamic-related molecules, and by upregulating AMPK activity in TPA-induced aggressive HT-29 cells.

PGC-1α is the main regulator of mitochondrial biogenesis, and acts through interactions with transcription factors such as NRF1 and ERRα (4). It is sensitive to cellular energy status, which is influenced by cold, exercise and fasting and its activity is closely controlled by several post-translational modifications.
such as phosphorylation and acetylation. Both AMPK-mediated phosphorylation and SIRT1-mediated deacetylation activate PGC-1α under energy deprivation conditions (21). NRF-1 regulates mtTFA expression, thereby mediating the increased expression of nuclear mitochondrial genes with increased mtDNA replication and expression (22,23). ERRα acts as a co-transcription factor of PGC-1α, and regulates the expression of genes involved in fatty acid oxidation, the TCA cycle, mitochondrial biogenesis and dynamics and OXPHOS. ERRα also acts both directly on genes involved in mitochondrial structural components and enzymes (24). Recent studies have revealed that the PGC-1α-mediated transcriptional cascade suppresses invasion and migration in different types of cancer cells. Integrative metabolomics and transcriptomics using genetically engineered mouse models and xenografts have shown that PGC-1α activates an ERRα-dependent transcriptional network to induce a catabolic state and suppress metastasis in prostate cancer (25). In melanoma, PGC-1α directly increases the transcription of DNA-binding protein inhibitor (ID2), which in turn binds to and inactivates transcription factor 4 (TCF4). TCF4 inactivation downregulates metastasis-related genes including integrins and integrin-mediated signaling pathways that play a role in invasion and migration (26). In the present study, we also found that proton beam irradiation effectively increased mitochondrial biogenesis by upregulating PGC-1α expression and its co-transcriptional factors such as NRF1 and ERRα, as well as the mitochondrial transcription factor mtTFA. Therefore, our findings demonstrate that increased mitochondrial biogenesis, dynamics, and function by a PGC-1α-mediated transcriptional cascade might be a novel mechanism underlying the inhibitory effects of proton beam irradiation on metastatic potential in TPA-induced aggressive HT-29 cells.

AMPK is a hetero-trimeric protein complex composed of a catalytic subunit and two regulatory subunits; it plays an essential role in regulating metabolic and energy homeostasis (27). In particular, AMPK is well known as the main regulator for lipid, cholesterol and glucose metabolism in various tissues, such as the liver, muscles and adipose tissues. Owing to this physiological role, AMPK has been considered a key therapeutic target in patients with metabolic diseases such as obesity and diabetes (28). Moreover, therapeutic manipulation for targeting AMPK signal pathways using some diabetes drugs in cancer patients shows the association between AMPK and several tumor suppressors (29). Recent studies have indicated that AMPK not only inhibits cancer cell proliferation but also invasion. The siRNA-mediated knockdown and pharmacologic activation of AMPK by OSU-53, a novel allosteric AMPK activator, suppresses the epithelial-mesenchymal transition (EMT) by modulating Akt-MDM2-Foxo3 signaling in breast and prostate cancer cells (30). Treatment with metformin, an AMPK activator, inhibits EMT in an AMPK/p53-dependent manner in metastatic melanoma cells (31) and inhibits both angiogenesis and metastatic spread in ovarian cancer (32). Similarly, Kim et al. (33) reported that AMPK activation by berberine, another AMPK activator, suppresses human melanoma cell migration by decreasing Erk activity and cytochrome c oxidase subunit II expression. Notably, our findings showed that proton beam irradiation increased TPA-inhibited AMPK phosphorylation, which supports the increased expression of PGC1-α and its co-transcription factors including NRF1 and ERRα, as well as target genes including Cytc, ATP5B and CPT-1α, which are associated with OXPHOS components and mitochondrial functional enzymes. Collectively, AMPK activation by proton beam irradiation is decisive for mitochondrial biogenesis, and may exert a tumor suppressor function and enhance mitochondrial function. However, the specific role of AMPK activation by proton beam irradiation on metastasis and mitochondrial function requires further study.

Mitochondria are highly dynamic organelles that are continuously undergoing fusion and fission; they are necessary for maintaining cellular physiological functions such as survival, death and metabolic homeostasis (34). Although mitochondrial dysfunction has been associated with tumorigenesis, the roles of mitochondrial dynamics in metastasis are poorly understood. Altered mitochondrial dynamics have been associated with changed mitochondrial physiology and cell dysfunction (35), which are involved in many human diseases such as cancer, neurodegenerative diseases including Alzheimer's, Huntington's and Parkinson's diseases, and cardiometabolic diseases including diabetes and cardiomyopathy (36). A recent study showed that human lung cancer cells have excess mitochondrial fission and impaired mitochondrial fusion due to imbalanced DRP1/MFN expression (37). Our findings suggest that proton beam irradiation effectively regulates dysfunctional mitochondrial dynamics in metastatic cancer by increasing the expression levels of DRP1 and OPA1.

Over 99% of proteins involved in mitochondrial biogenesis are imported from the cytoplasm. This mitochondrial protein import process occurs through channel protein complexes such as the translocase of the outer membrane (TOM) and the translocases of the inner membrane (TIM) (38). Mitochondrial membrane channels such as ion and protein channels are considered promising therapeutic targets for cancer transformation owing to their correlation in metabolic and apoptotic functions (39). In particular, many subunits of the mitochondrial protein import complexes are dysregulated in cancer cells (40). The mitochondrial protein import system subunits TIMM44 and TOM40 play an important role in mitochondrial functions such as the import of OXPHOS-related proteins and mitochondrial redox balance (41). Our findings show that proton beam irradiation might be an effective regulator of TIMM44 and TOM40 expression.

In conclusion, our findings show that proton beam irradiation increases mitochondrial biogenesis by modulating the expression of the main mitochondrial transcription factors, functional regulators and dynamic-related molecules by inducing AMPK activity in TPA-induced aggressive HT-29 cells. We propose that targeting mitochondrial biogenesis may be a novel inhibitory mechanism for metastatic cancer metabolism.

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