Loss of mitochondrial DNA enhances angiogenic and invasive potential of hepatoma cells

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Received July 24, 2009; Accepted October 2, 2009

DOI: 10.3892/or_00000698

Abstract. Mitochondrial DNA (mtDNA) mutations are frequently found in a variety of tumors. However, the role of mtDNA mutations in tumor behavior is poorly understood. We explored the effects of mtDNA mutations on tumor phenotype employing mtDNA-depleted SK-Hep1 ρ^0 hepatoma cells. Expression of hypoxia inducible factor (HIF)-2α mRNA was markedly increased in ρ^0 cells compared to control cells. Protein level of HIF-2α was increased in SK-Hep1 ρ^0 cells compared to control cells in hypoxic but not in normoxic conditions, suggesting that mitochondrial dysfunction increases angiogenic potential of tumor cells. Expression of HIF-2α was increased at the RNA level after treatment of SK-Hep1 hepatoma cells with ethidium bromide (EtBr) or inhibitors of mitochondrial complexes. HIF reporter activity and the expression of vascular endothelial growth factor (VEGF), an angiogenic key molecule induced by HIF, were increased in SK-Hep1 ρ^0 cells compared to their normal counterparts. Tube formation assay and chick chorioallantoic membrane (CAM) assay showed that conditioned medium (CM) from mtDNA-depleted SK-Hep1 ρ^0 cells increased formation of tube-like structures and new blood vessels relative to that from control cells. In SK-Hep1 ρ^0 cells, expression of genes related to invasion such as urokinase-type plasminogen activator (uPA) or matrix metalloproteases (MMPs) was also upregulated compared to control cells, suggesting that mitochondrial dysfunction could also increase invasive potential of tumor cells. These results strongly suggest that HIF-2α mRNA expression is increased in tumor cells with mtDNA mutations or deletions, which contributes to the angiogenic and invasive potential of tumor cells.

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Key words: mtDNA, hypoxia inducible factor-2α, vascular endothelial growth factor, angiogenesis

Introduction

According to Warburg's effect, glycolysis is increased in a variety of tumor cells as a source of energy even if oxygen is abundant. The Warburg's effect suggests that mitochondrial respiration in tumor cells might be defective, which could lead to increased glycolysis (1). Consistent with such a hypothesis, previous studies have reported frequent point mutations or deletions in mitochondrial DNA (mtDNA) in diverse types of tumor cells such as gastric, colon and lung cancer cells (2). Further, the expression and activity of mitochondrial enzymes for oxidative phosphorylation were reduced in such cancer cells compared to normal controls. Based on those findings, it has been previously suggested that defects in mitochondrial function may play a role in tumorigenesis (3-6). We also reported that MTA1 or antioxidant enzymes overexpressed in mtDNA-depleted tumor cells might lead to enhanced angiogenesis or resistance to various types of cell death, supporting that mtDNA mutations might be related to the malignant potential of cancer cells (7-9). However, the cellular or biochemical mechanism of the increased tumorigenicity by mtDNA mutations or deletions is far from clear.

As the tumor volume increases, tumor cells struggle to remodel their environment and display a series of adaptive responses since they are exposed to hypoxia and energy starvation, especially in the central area of the tumor mass. For instance, tumor cells secrete angiogenic factors such as vascular endothelial growth factor (VEGF) to their surrounding stromal tissues to increase the formation of blood vessels and to match the metabolic demand for oxygen and nutrients (10). Hypoxia-inducible factor (HIF), a master transcriptional factor for angiogenesis, accumulates in hypoxic conditions and plays a pivotal role in regulating transcription of VEGF in tumor cells (11). Such increased angiogenesis by HIF may also participate in the tumor cell spreading from primary site to distant organs (12). It is also possible that HIF in tumor cells regulate invasive potential of tumor cells by inducing molecules related to the degradation of extracellular matrix (ECM) (13,14).

SK-Hep1 ρ^0 cells derived by treating parental cells with ethidium bromide (EtBr) or other agents that inhibit mtDNA replication are defective in various mitochondrial functions (15) and useful for the study of the role of mitochondria and nuclear-mitochondrial crosstalk (16,17). We report herein that expression of HIF-2α is increased in mtDNA-depleted SK-Hep1 ρ^0 cells, which leads to the induction of VEGF and enhanced angiogenic activity. These results suggest that mitochondrial mutations or deletions frequently detected in tumor cells might contribute to the tumor progression by increasing their angiogenic, invasive and metastatic potential.

Materials and methods

Cell culture. SK-Hep1 cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37˚C in 5% CO_2. To make mtDNA-depleted ρ^0 cells, SK-Hep1 hepatoma cells were cultured in the presence of 100 ng/ml EtBr (Sigma, St. Louis, MO, USA) for >20 generations and maintained in the presence of 50 μg/ml uridine (Sigma). The ρ^0 cells used in this study were authentic mtDNA-depleted cells as described (9).

RT-PCR. Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNAs were synthesized using Superscript II and oligo$(dT)_{12-18}$ primer (Invitrogen) according to the manufacturer's protocol. The PCR primer sequences were: ß-actin sense, 5'-TGACGGG GTCACCCACACTGTGCC-3'; antisense, 5'-TAGAAGCA TTTGCGGTGGACGATG-3'; HIF-1α sense, 5'-CTGAC CCTGCACTCAATCAA-3'; antisense, 5'-CTTTGAGGA CTTGCGCTTTC-3'; HIF-2α sense, 5'-AGCAGCTGGA GAGCAAGAAG-3'; antisense, 5'-GCTTCAGCTTGTT GGAGAGG-3'; matrix metalloproteinase (MMP)-2 sense, 5'-GCGACAAGAAGTATGGCTTC-3'; antisense, 5'-TGC CAAGGTCAATGTCAGGA-3'; MMP-9 sense, 5'-CGCAG ACATCGTCATCCAGT-3'; antisense, 5'-GGATTGGC CTTGGAAGATGA-3'; membrane type 1-matrix metalloproteinase (MT1-MMP) sense, 5'-CCCTATGCCTACATC CGTGA-3'; antisense, 5'-TCCATCCATCACTTGGTTAT-3'; VEGF sense, 5'-GAGAATTCGGCCTCCGAAACCATG AACTTTCTGCT-3'; antisense, 5'-GAGCATGCCCTCCTG CCCGGCTCACCGC-3'.

Western blotting. Cells were lysed in buffer containing 50 mM Tris-HCl (pH 7.6), 250 mM NaCl, 1% Nonidet P-40, 0.1% SDS and protease inhibitors (Roche Applied Science, Mannheim, Germany). After centrifugation to remove insoluble material, proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% nonfat dry milk in Tris-buffered saline/0.1% Tween-20 at 4˚C for 1 h, and then incubated with primary antibodies such as anti-HIF-1α (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-HIF-2α (Novus Biologicals, Littleton, CO, USA), anti-MMP-2 (Oncogene Science, Cambridge, NY, USA) or anti-MMP-9 antibody (Oncogene Science) overnight at 4˚C. After incubation with the corresponding horseradish peroxidase-labeled secondary antibodies, proteins were visualized using an enhanced chemiluminescence kit (Amersham Biosciences, Arlington Heights, IL, USA).

Reporter assay. Cells were transfected with 0.5 μg of pGL3 firefly luciferase reporter plasmid containing the hypoxia-response element (HRE) sequence that is located in the erythropoietin gene promoter (pGL3-EPO-HRE-LUC) and 0.01 μg of pRL-CMV *Renilla* luciferase plasmid using polyethylenimine (Sigma) as a gene carrier. After 48 h of incubation, the cells were exposed to hypoxia. Firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase reporter assay system (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions.

Preparation of serum-free conditioned medium (CM). When cells were subconfluent, serum-supplemented medium was removed and the cell monolayer was washed with PBS to remove remaining serum proteins. The cells were then cultured in serum-free DMEM without any other protein for 24 h. The CM was loaded onto Centricon filter devices with a cutoff of 3 kDa (Millipore, Bedford, MA, USA) and concentrated by centrifugation according to the manufacturer's instructions. Aliquots were frozen at -70˚C until use.

Tube formation assay. Growth factor-reduced Matrigel (BD Biosciences, San Jose, CA, USA) was placed in a 24-well culture plate at 10 mg/ml and polymerized for 1 h at 37˚C. Human umbilical vein endothelial cells (HUVECs) were seeded in each well at $1.5x10^5$ cells/well together with concentrated CM (50 x) or VEGF (20 ng/ml) as a positive control. After 24 h, tube formation was assessed by inverted phase-contrast microscopy. The degree of tube formation was quantified by measuring the mean length of tubes in three randomly chosen fields from each well using Image J software (NIH Image, Bethesda, MD, USA).

Chorioallantoic membrane (CAM) assay. The CAM assay was performed using 9-day-old chick embryos as previously described (7).

Zymography analyses. For gelatin zymography or casein/plasminogen zymography, CM of cultured cells was solubilized in a sample buffer lacking reducing agents. Samples were electrophoresed in SDS-polyacrylamide gels containing 2 mg/ml gelatin (Sigma) or 1 mg/ml α-casein (Sigma)-10 μg/ml plasminogen (Sigma). After electrophoresis, the gels were rinsed twice in 2.5% Triton X-100 at room temperature and then incubated in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 10 mM $CaC1_2$, 2 μM $ZnCl_2$, 0.002% NaN_3, 1% Triton X-100 overnight at 37˚C. Subsequently, the gels were stained with Coomassie brilliant blue and then destained. Gelatinolytic activity resulting from MMP activation or caseinolytic activity resulting from urokinase-type plasminogen activator (uPA) was identified as white zone after staining with Coomassie brilliant blue.

Results

Increased HIF-2α expression in mtDNA-depleted ρ^0 cells. In our previous study, we studied the expression of various

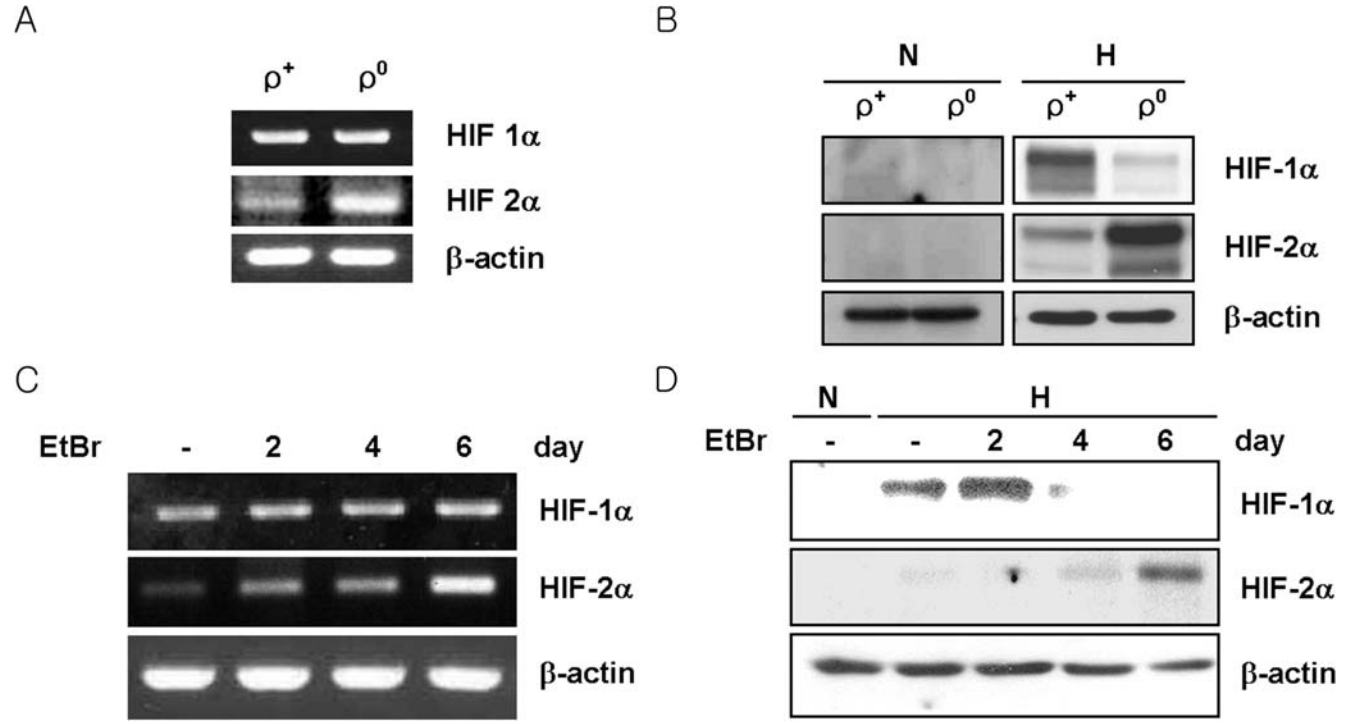

Figure 1. Effect of mtDNA depletion on the expression of HIF-1α and HIF-2α. (A) mRNA levels of HIF-1α, HIF-2α and ß-actin were determined by RT-PCR in SK-Hep1 ρ^0 and control cells maintained in normoxic conditions. (B) Cells were incubated in normoxic (20% O_2) or hypoxic (1% O_2) conditions for 6 h, and protein levels of HIF-1α, HIF-2α and ß-actin were examined by Western blot analysis. N, normoxia; H, hypoxia. (C) Cells were incubated with EtBr, an inhibitor of mtDNA replication and transcription, for the indicated time periods. mRNA expression of HIF-1α and HIF-2α was examined by RT-PCR, which was normalized to the ß-actin mRNA expression. (D) Cells were incubated with EtBr for the indicated time periods and then exposed to normoxic or hypoxic conditions for 6 h. Protein expression of HIF-1α and HIF-2α were analyzed by Western blotting, which was normalized to the ß-actin protein expression.

genes in mtDNA-depleted SK-Hep1 ρ^0 hepatoma cells using DNA chip analysis (Genomic Tree, Taejon, Korea) (9). Among them, HIF-2α was one of the genes that were most highly upregulated in SK-Hep1 ρ^0 cells compared to parental cells (data not shown). RT-PCR analysis confirmed the increased expression of HIF-2α mRNA in SK-Hep1 ρ^0 cells compared to parental cells (Fig. 1A). In contrast, the expression of HIF-1α mRNA was not different between SK-Hep1 ρ^0 and parental cells. We next examined whether the changes in mRNA expression lead to the changes of protein expression. Western blotting showed no detectable expression of HIF-1α or HIF-2α protein in both SK-Hep1 ρ^0 and parental cells grown in normoxic conditions, most likely because of rapid degradation of HIF proteins in normoxia (Fig. 1B) (18). In contrast, a significant accumulation of HIF-2α protein was observed in SK-Hep1 ρ^0 cells grown in hypoxic conditions, while HIF-2α protein was undetectable in parental cells cultured in the same conditions (Fig. 1B). HIF-1α protein was induced in parental cells after hypoxic culture; however, HIF-1α protein induction was less prominent in SK-Hep1 ρ^0 cells, suggesting that HIF-2α protein expression was selectively upregulated in mtDNA-depleted SK-Hep1 ρ^0 cells and HIF-1α protein was less well stabilized in ρ^0 cells compared to parent cells probably due to increased availability of cytoplasmic oxygen and prolyl hydroxylation of HIF-1α in SK-Hep1 ρ^0 cells (19).

Next, we studied whether disruption of mitochondrial function by mitochondrial inhibitors induces similar changes in the expression of HIF. When the parental SK-Hep1 hepatoma cells were incubated with EtBr that inhibits the replication and transcription of mtDNA but has a negligible effect on nuclear DNA, the expression of HIF-2α mRNA measured by RT-PCR increased progressively up to day 6 in a time-dependent manner (Fig. 1C). Western blotting also showed that a gradual increase of HIF-2α protein in SK-Hep1 cells after treatment with EtBr in hypoxic conditions (Fig. 1D). In contrast, HIF-1α expression was not increased by EtBr treatment at the RNA or protein levels (Fig. 1C and D). In fact, the HIF-1α protein level after hypoxic culture was decreased by EtBr, consistent with the attenuated induction of HIF-1α protein in SK-Hep1 ρ^0 cells compared to parental cells (19).

Increased expression of VEGF in mtDNA-depleted ρ^0 cells. We then studied the transcriptional activities of HIF in SK-Hep1 ρ^0 cells and control cells cultured in hypoxic conditions, since HIF proteins are master transcriptional factors regulating the expression of a variety of genes in hypoxia. The promoter assay after transfection of a reporter gene containing HRE sequences showed that HRE reporter activity was apparently increased in both SK-Hep1 ρ^0 cells and parental cells in hypoxic culture compared to those in normoxia. However, the increase in HRE transcriptional activity was significantly more pronounced in SK-Hep1 ρ^0 cells compared to parental cells, probably because transactivation by the increased HIF-2α expression in SK-Hep1 ρ^0 cells was greater than that by

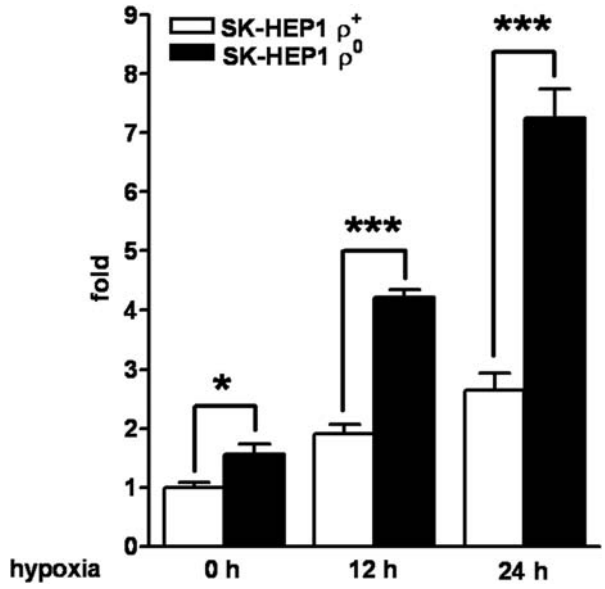

Figure 2. Effect of mtDNA depletion on HRE reporter activity. Cells were transfected with pGL3-EPO-HRE-LUC plasmid and pRL-CMV *Renilla* luciferase plasmid. After incubation in normoxic or hypoxic conditions for 12 or 24 h, luciferase activities were measured. The data are presented as the mean ± SEM of three independent experiments. (*P<0.05; ***P<0.001).

increased HIF-1α expression in control cells (P<0.05 at 0 h and P<0.001 at 12 and 24 h by ANOVA) (Fig. 2).

Next, we explored whether increased HRE transcriptional activity in SK-Hep1 ρ^0 cells grown in hypoxia leads to enhanced expression of HIF target proteins related to angiogenesis. RT-PCR analysis showed that mRNA expression of VEGF, a prototypic HIF target protein, was increased after hypoxic culture for 24 h in both parental and SK-Hep1 ρ^0 cells (Fig. 3A). At the protein level also, Western blotting showed increased VEGF expression in both parental and SK-Hep1 ρ^0 cells cultured in hypoxic conditions (Fig. 3B). Herein, the increments of VEGF mRNA and protein expression were apparently more pronounced in SK-Hep1 ρ^0 cells compared to parental cells grown in the same conditions, which was probably due to the contribution of both HIF-2α mRNA induction and hypoxia in SK-Hep1 ρ^0 cells compared to that of hypoxia alone in parental cells and suggests that increased HRE transcriptional activity by HIF-2α protein induction in ρ^0 cells leads to the significantly increased expression of HIF target proteins.

To further investigate the effects of mitochondrial dysfunction on the expression of HIF-2α mRNA, we used pharmacologic inhibitors of the mitochondrial respiratory chain. When SK-Hep1 hepatoma cells were treated with rotenone (a complex I inhibitor), antimycin A (a complex III inhibitor), DETA-NO (a complex IV inhibitor), HIF-2α mRNA level was increased as shown by RT-PCR analysis, directly demonstrating that interruption of mitochondrial respiration leads to an increased expression of HIF-2α at the RNA level. In contrast, no significant change in HIF-1α mRNA level was seen after treatment with mitochondrial complex inhibitors (Fig. 4), suggesting selective HIF-2α mRNA induction by mitochondrial dysfunction.

Induction of angiogenesis by mtDNA-depleted ρ^0 cells. We next studied whether the increased expression of angiogenic molecules such as VEGF in SK-Hep1 ρ^0 cells could indeed induce angiogenesis. The tube formation assay showed that CM from SK-Hep1 ρ^0 cells induced an enhanced tube-like extension of HUVECs grown on Matrigel compared to control CM from parental cells (Fig. 5A). The average length of the tube network was significantly longer after treatment with

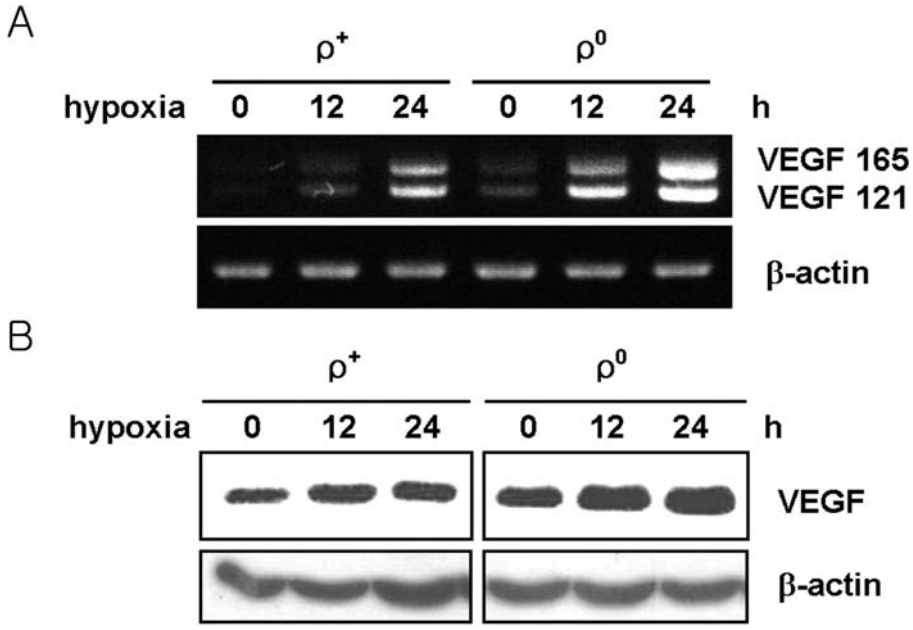

Figure 3. Effect of mtDNA depletion on the expression of VEGF. (A) mRNA levels of VEGF were assessed in cells incubated in normoxic or hypoxic conditions for the indicated time periods by RT-PCR. (B) The protein levels of VEGF were also examined in cells cultured in normoxic or hypoxic conditions for the indicted time periods by Western blotting.

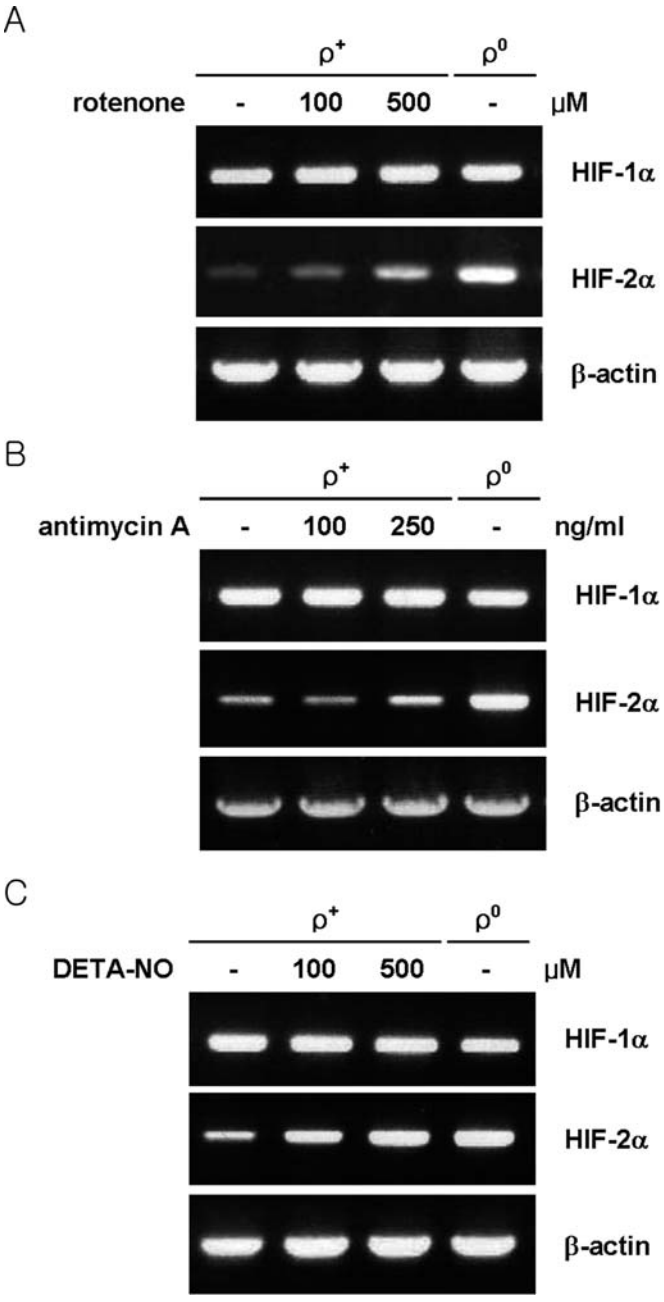

Figure 4. Effect of mitochondrial complex inhibitors on the expression of HIF-1α and HIF-2α mRNA. The expression of HIF-1α and HIF-2α was studied in cells exposed to increasing concentrations of inhibitors of the electron transport chain by RT-PCR.

CM from SK-Hep1 ρ^0 cells compared to that after treatment with CM from control cells ($P<0.01$). CAM assay also showed that treatment of CAM with concentrated CM from SK-Hep1 ρ^0 cells resulted in significant increases in the density of vessels *in vivo* compared to that with control CM from parental cells (Fig. 5B), suggesting that tumor cells with mitochondrial dysfunction are able to induce angiogenesis by secreting angiogenic factors such as VEGF.

ECM degradation by mtDNA-depleted cells. We next examined expression of genes that are potentially regulated by HIF (20) and involved in the behavior of cancer cells such as invasiveness or metastasis because our DNA chip data showed the increased expression of MMPs and uPA in SK-Hep1 ρ^0 cells (data not shown). RT-PCR and Western blotting confirmed increased expression of MMP-2, MMP-9 and MT1-MMP in SK-Hep1 ρ^0 cells compared to parental cells (Fig. 6A and B). Zymography also showed that CM from SK-Hep1 ρ^0 cells had increased gelatinolytic activity of MMP-2 and MMP-9, and increased caseinolytic activity of uPA that are involved in ECM degradation, compared to that from parental cells (Fig. 6C and D).

Discussion

In the present study, we asked whether the mitochondrial dysfunction contributes to the widely described alterations in the behavior of tumor cells such as increased angiogenesis or invasiveness because our chip data showed increased expression of molecules related to such abnormal behavior in mtDNA-depleted SK-Hep1 ρ^0 cells. We observed that in mtDNA-depleted SK-Hep1 hepatoma cells, the mRNA of oxygen sensing transcription factor HIF-2α was overexpressed compared to control cells. In hypoxic conditions, level of HIF-2α protein expression was also markedly higher in SK-Hep1 ρ^0 cells compared to control cells, whereas expression of HIF-1α protein was not increased in ρ^0 cells. The HRE reporter activity reflecting HIF-dependent transactivation was also increased to a greater degree in SK-Hep1 ρ^0 cells compared to parental cells, which is probably due to increased HIF-2α protein expression. The expression of VEGF, an angiogenic key molecule mainly induced by HIF, was also more pronounced in SK-Hep1 ρ^0 cells compared to parental cells. Therefore, the elevated expression of HIF-2α may be responsible for the overall transcriptional activity of hypoxia-responsive genes such as VEGF in SK-Hep1 ρ^0 cells.

HIF expression is usually regulated by post-translational mechanism. Hypoxia increases the expression level of HIF protein by blocking the VHL-dependent proteasomal degradation through the inhibition of prolyl hydroxylase. However, in the case of mtDNA-depleted ρ^0 cells or cells treated with mitochondrial inhibitors, expression of HIF was regulated by transcription modulation. The mechanism of increased mRNA expression of HIF-2α is not clear. Some previous studies reported that the expression of HIF-α can be modulated at the transcriptional level by Rho GTPase (21), lipopolysaccharide (22) or Nox4 (23). While Nox4 was overexpressed in SK-Hep1 ρ^0 cells, Nox inhibitors such as apocynin and DPI or antioxidants did not affect HIF-2α mRNA expression (data not shown). Other mechanisms potentially related to mRNA expression of HIF such as CCAAT/enhancer binding protein ß might be involved in the increased HIF-2α expression in SK-Hep1 ρ^0 cells, while such possibilities were not pursued further in this investigation (24,25).

Increased mRNA expression of HIF-2α is expected to increase tumor angiogenesis in conjunction with tumor hypoxia as was demonstrated in our results showing increased expression of HIF-2α protein after hypoxic culture of SK-Hep1 ρ^0 cells. However, expression of HIF-1α protein after hypoxic culture was less prominent in SK-Hep1 ρ^0 cells compared to wild-type cells (19). In the case of HIF-2α, profoundly increased mRNA expression might surpass the effect of increased cytoplasmic O_2 availability in such cells.

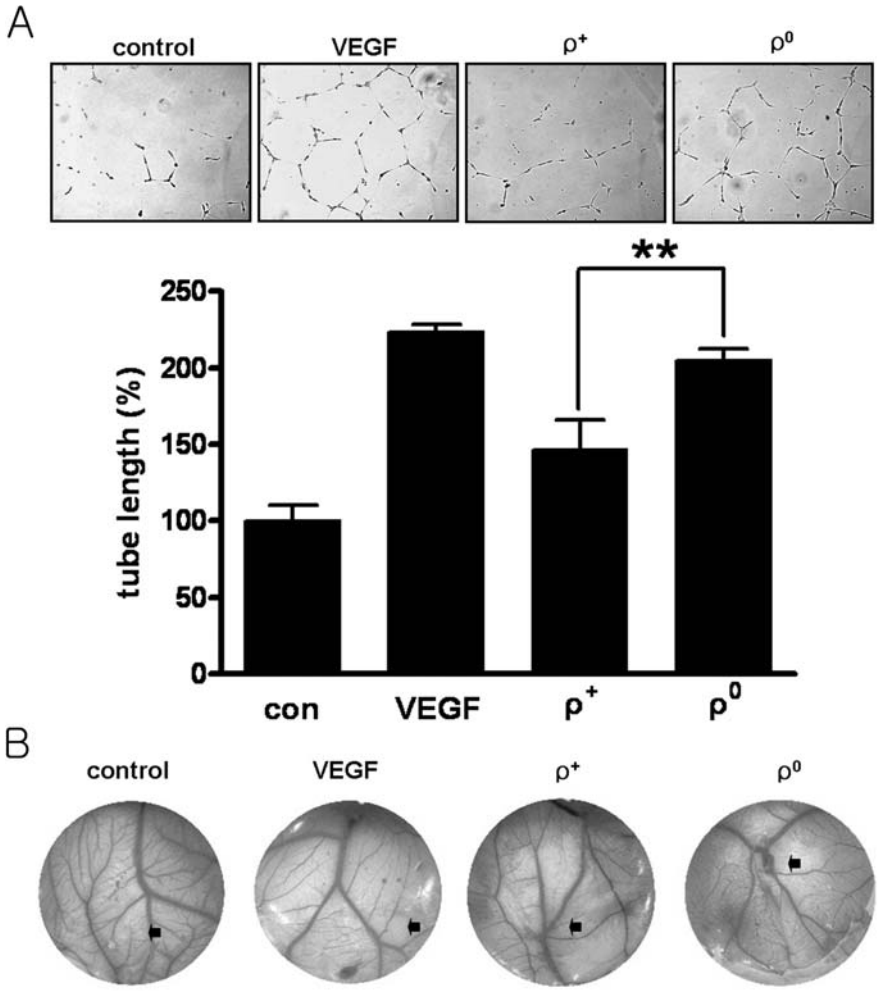

Figure 5. Effect of mtDNA depletion on angiogenesis. (A) HUVECs were seeded on the Matrigel and incubated with concentrated CM or VEGF as a positive control for 24 h to assess *in vitro* tube formation. The data are presented as the mean ± SEM of three independent experiments ($^{**}P<0.005$). (B) Nine-day-old CAMs were treated with concentrated CM or VEGF as a positive control for 3 days. Black arrows indicate the locations of Thermanox coverslips to which the reagents were loaded.

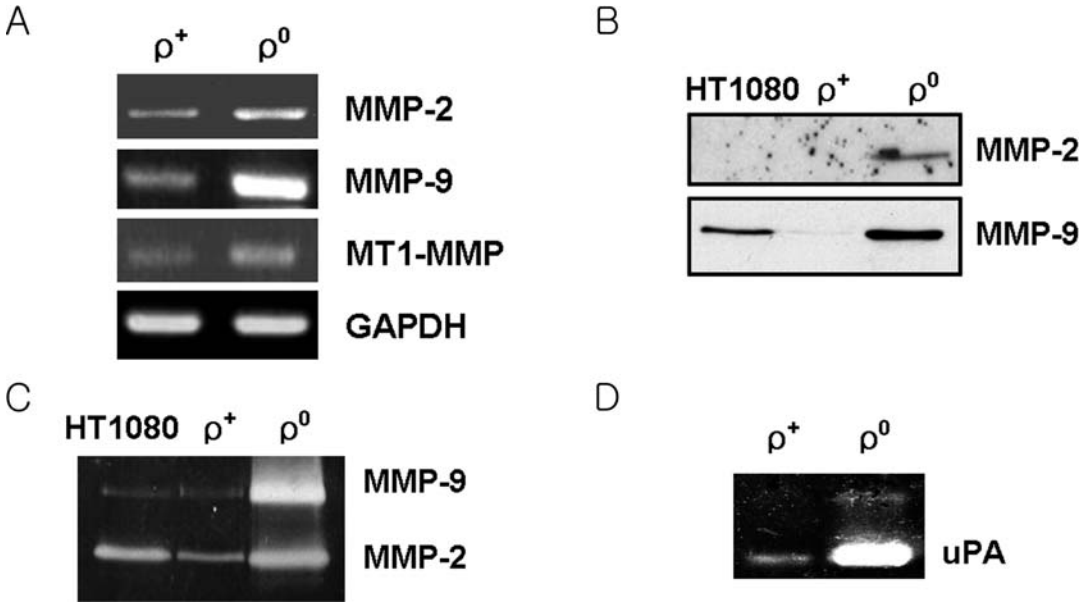

Figure 6. Effect of mtDNA depletion on the expression and activity of MMPs and plasminogen activators. (A) RT-PCR was performed to assess expression of MMP-2, MMP-9 and MT1-MMP mRNA. (B) CM was obtained from cells cultured in serum-free media for 24 h and then concentrated with a Centricon filter. The proteins in CM were analyzed by Western blotting using anti-MMP-2 or anti-MMP-9 antibodies. (C and D) The activities of MMP-2, MMP-9 and uPA in concentrated CM were assessed by gelatin and casein/plasminogen zymography, respectively.

While we observed selective induction of HIF-2α mRNA and protein in mtDNA-depleted hepatoma cells, other investigators reported increased expression of HIF-1α in cybrid cells that carry mutated mtDNA from highly metastatic tumors (3,26), suggesting that pattern of HIF induction might be cell type-dependent.

An important characteristic of malignant tumors is their invasive potential and ability to induce remodeling of connective tissue by upregulated production and secretion of MMPs and plasminogen activators (27-29). MMP-2 and MMP-9 are the gelatinases that preferentially degrade type IV collagen, a major component of the basement membrane, and MT1-MMP can degrade pericellular ECM and is crucial for activation of MMP-2 (30). MMPs release the cell-membrane-bound precursors of some growth factors, and cleave VEGF or other growth factors sequestered by ECM (31-33). uPA, a serine protease, converts plasminogen to plasmin, which directly mediates invasion by degrading ECM and indirectly by activating latent forms of MMPs (27). It is well documented that MMPs and the uPA/plasmin system are associated with invasive or metastatic potential of several solid tumor types and their prognosis (28,29). Furthermore, certain MMPs have been reported to be induced by hypoxia in a HIF-dependent manner (13,14), suggesting that tumor cells in hypoxic conditions may endeavor to move to the area where the metabolic environment is more favorable by upregulation of the enzymes degrading ECM. Therefore, our data showing increased expression and activity of MMPs and uPA in mtDNA-depleted cells suggest the possibility that mtDNA mutations could be a mechanism by which cancer cells acquire invasive or metastatic potential, which is consistent with previous studies showing increased expression of cathepsin or genes involved in ECM remodeling such as MMP-1, TIMP and uPA after treatment of tumor cells with mitochondrial uncouplers, mitochondrial complex inhibitors or EtBr (34-36).

Tumor cells with mitochondrial dysfunction depend on anaerobic respiration since their electron transport chain utilizing oxygen was impaired. Hence, they may recognize themselves as being in hypoxia-like conditions and may try to escape from their predicament by modulating the expression of diverse nuclear genes. Upregulation of genes related to angiogenesis or invasion could be a mechanism for the enhanced survival of tumor cells with mitochondrial mutations or deletions, and HIF-2α could be an important factor regulating the induction of such genes.

Acknowledgements

This work was supported by the 21C Frontier Functional Proteomics Project of the Korean Ministry of Science and Technology (FPR08B1-210) and Nano/Bio Science Program Grant (F104AC010002-08A0301-00220). M.-S. Lee is the recipient of the Samsung Biomedical Research Institute Grant (C-A6-421-1) and the Bio R&D program of the National Research Foundation of Korea (2008-04090).

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