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Serum Deprivation Enhances Apoptotic Cell Death by Increasing Mitochondrial Enzyme Activity

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Abstract – Mitochondria are important sensor of apoptosis. H_2O_2-induced cell death rate was enhanced by serum deprivation. In this study, we investigated whether serum deprivation using 0.5 or 3 % FBS induces apoptotic cell death through mitochondrial enzyme activation as compared to 10 % FBS. Apoptotic cell death was observed by chromosome condensation and the increase of sub-G0/G1 population. Serum deprivation reduced cell growth rate, which was confirmed by the decrease of S-phase population in cell cycle. Serum deprivation significantly increased caspase-9 activity and cytochrome *c* release from mitochondria into cytosol. Serum deprivation-induced mitochondrial changes were also indicated by the increase of ROS production and the activation of mitochondrial enzyme, succinate dehydrogenase. Mitochondrial enzyme activity increased by serum deprivation was reduced by the treatment with rotenone, mitochondrial electron transport inhibitor. In conclusion, serum deprivation induced mitochondrial apoptotic cell death through the elevation of mitochondrial changes such as ROS production, cytochrome *c* release and caspase-9 activation. It suggests that drug sensitivity could be enhanced by the increase of mitochondrial enzyme activity in serum-deprived condition.

Keywords □ Serum deprivation, Mitochondria, ROS, HeLa cells, Apoptosis

INTRODUCTION

The apoptotic pathways are regulated by multiple signaling processes. Many proapoptotic agents induce chromatin condensation and large scale DNA fragmentation in isolated nuclei (Susin, *et al.*, 1999[a]; Susin, *et al.*, 1999[b]). Two main apoptotic pathways have been elucidated, which require the participation of cysteine-aspartate proteases (caspases) (Minamino, *et al.*, 2002). The first of these apoptotic pathways involves death receptors, while the second of the pathways involves the mitochondria.

Mitochondria are important sensor and/or executioner of apoptosis (Susin, *et al.*, 1999[b]). Mitochondria-dependent apoptotic pathway is activated by the release of proapoptotic molecules including cytochrome *c* and apoptosis inducing factor (AIF) from mitochondria (Joza, *et al.*, 2001). Cytochrome *c* forms a complex, termed "apoptosome," with apoptosis-activating factor (Apaf-1) and procaspase-9, which resulted in the activation of caspase-9 and a subsequent caspase cascade (Royall and Ischiropoulos, 1993).

Serum deprivation induced apoptosis in many types of cells, such as cardiac myocytes (Bialik, et al., 1999), neuronal precursors (Colombaioni, *et al.*, 2002[a]; Colombaioni, *et al.*, 2002[b]), tumor cells (Kazi and Dou, 2005), and mesenchymal stem cells (MSCs) (Zhu, *et al.*, 2006). Tumor cell death by anticancer drugs was synergistically increased by serum deprivation to synchronize human tumor cells in specific phases of the cell cycle (Kazi and Dou, 2005). The mitochondrial apoptotic pathway can be activated by serum deprivation, staurosporine treatment, and oxidative stress (Roucou, *et al.*, 2001). In hippocampal neuroblasts, serum deprivation triggered apoptosis through Ca^{++} mobilization from ER and caspase-12 activation that may constitute an amplifying loop of the mitochondrial pathway (Voccoli, *et al.*, 2007). Serum deprivation-induced apoptosis was increased by PI3K/Akt and MEK/ERK1/2 pathways (Xu, *et al.*, 2008) and inhibited by p38/Bcl-2 pathways (Nelyudova, *et al.*, 2007). However, little has been known about whether serum deprivation induces apoptotic cell death through the activation of mitochondrial electron transport and mitochondrial enzyme.

The aims of this study were to investigate the involve-

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ment of the mitochondrial enzyme activation in the induction of apoptosis after prolonged serum deprivation of HeLa cervical tumor cells.

MATERIALS AND METHODS

Reagents

Monoclonal anti-tubulin antibodies were from Sigma; cytochrome *c* antibodies and HRP-conjugated goat anti-mouse or rabbit IgG were from Amersham; polyclonal anti-caspase-3 antibodies were from StressGen; N-acetyl (Ac)-DEVD-*p*-nitroanilide (NA) was from Calbiochem; N-Ac-LEHD-*p*-NA was from Biomol. Except where indicated, all other materials are obtained from the Sigma chemical company (St. Louis, MO).

Cell culture

HeLa cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine and penicillin/streptomycin. The cells were incubated in a 5 % CO_2 at 37°C. For the measurement of cell survival rate with MTT, 20,000 cells were seeded in 96-well plate for 4 hrs. Then, the cells were incubated with 0.5, 3 and 10% of FBS in the presence or absence of various concentrations of H_2O_2 for appropriate time. For the measurement of mitochondrial enzyme activity, 5×10^3, 7.5×10^3, 1.0×10^4 cells were seeded in 96-well plate with 0.5 , 3, 10% FBS, respectively. Then, the cells were cultured for appropriate days.

Immunostaining

Cells were fixed in 3% paraformaldehyde for 30 minute. Nuclei were detected by staining with DAPI. Cells were observed with a magnification of 400X in fluorescence microscopy (Nikon, Japan).

MTT assay

We quantified cell survival using colorimetric assay described for measuring intracellular succinate dehydrogenase content with MTT [3(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Denizot and Lang, 1986; Slater, *et al.*, 1963). Confluent cells were cultured with various concentrations of H_2O_2 for 48 hour. Cells were then incubated with 50 μg/ml of MTT at 37°C for 2 hour. Dissolved MTT was converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes (Slater, *et al.*, 1963). Formazan formed were dissolved in dimethylsulfoxide (DMSO). Optical density (OD) was read at 540 nm.

Flow cytometry analyses

Hypodiploid cell formation and the production of reactive oxygen species (ROS) were analyzed by flow cytometry that was performed with CELLQuest™ software in FACS-calibur™ (Becton Dickinson, San Jose, CA). For the determination of hypodiploid cell formation, cells were fixed in 40% ethanol on ice for 30 minutes and then incubated with propidium iodide (PI, 50 μg/ml) and RNase (25 μg/ml) at 37°C for 30 minutes. 10,000 cells were analyzed using flow cytometry.

ROS level was determined by incubating the cells with 10 μM 2, 7-dichlorodihydrofluorescein diacetate (DCF-DA) for 15 minutes at 37°C (Royall and Ishiropoulos, 1993; McLennan and Degli, 2000). The cells were detached by the incubation with trypsin/EDTA (GIBCO) for an additional 2 minutes at 37°C. Fluorescence intensity of 10,000 cells was analyzed using flow cytometry.

Separation of heavy-membrane and cytosolic fractions

HeLa cells were suspended in hypotonic buffer [20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES, pH 7.5), 10 mM $MgCl_2$, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 1 mM Na_4VO_3, 250 mM sucrose, and proteinase inhibitors]. After sonication [Fisher Model 550 (Pittsburgh, PA), 20% power, ten 1-second cycles with 50% elapsed time], supernatant containing cytosol fraction was obtained by centrifugation at 10,000*g* for 20 minute at 4°C. The pellet fraction containing mitochondria was dissolved in cell lysis buffer and then incubated for 30 minutes on ice before centrifugation at 14,000 rpm for 5 minute at 4°C.

Caspase-3-like and caspase-9-like activity assay

The colorimetric assay was carried out by means of a previously described protocol (Moon, *et al.*, 2004; Moon and Lerner, 2003). Briefly, caspase-3-like and caspase-9-like activity was measured in the mixture of assay buffer [100 mM HEPES, (pH 7.5), 5 mM EDTA, 0.1% (3-cholamidopropyl)-dimethylammonio)-1-propane-sulfonic acid (CHAPS), 5 mM DTT, and 20% glycerol], cell lysates, and 100 mM N-Ac-DEVD-*p*-NA for caspase-3 and 100 mM N-Ac-LEHD-*p*-NA for caspase-9. After incubation for 1 hour, absorbance was read at 405 nm. According to the manu-

facturer's instruction, the caspase-3 substrate can also be cleaved by caspases-6, -7, -8, and -10 and the caspase-9 substrate by caspases-4 and -5.

Western blot analysis

Cells were lysed in ice-cold lysis buffer, containing 0.5% Nonidet P-40 (vol./vol.) in 20 mM Tris-HCl (pH 8.3); 150 mM NaCl; protease inhibitors (2 μg/ml aprotinin, pepstatin, and chymostatin; 1 mg/ml leupeptin and pepstatin; 1 μM phenylmethyl sulfonyl fluoride (PMSF)) and 1 mM Na_4VO_3. Lysates were incubated for 30 minute on ice prior to centrifugation at 14,000 rpm for 5 minute at 4°C. Proteins in the supernatant were denatured by boiling for 5 minute in sodium dodecyl sulfate (SDS) sample buffer. Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to nitrocellulose membranes. Following this transfer, equal loading of protein was verified by Ponceau staining. The membranes were blocked with 5% skim milk in Tris-buffered saline with Tween 20 (TBST) (10 mM Tris-HCl, pH 7.6; 150 mM NaCl; 0.5% Tween 20), and then incubated with the indicated antibodies. Bound antibodies were visualized with HRP-conjugated secondary antibodies with the use of enhanced chemiluminescence (ECL) (Amersham, Piscataway, NJ).

Statistical analyses

Experimental differences were tested for statistical significance using ANOVA and Students' *t*-test. *P* value of < 0.05 or < 0.01 was considered to be significant.

RESULTS

Serum deprivation enhanced H_2O_2-induced cell death

Given that higher basal level of ROS is one of the mechanisms determining a higher apoptotic susceptibility of acute promyelocytic leukemia cells, NB4 cells, to antitumor agent-induced apoptosis (Wang, *et al.*, 2007[a]), HeLa cells were treated with various concentrations of H_2O_2 in the presence of 0.5, 3, 10% fetal bovine serum (FBS). Cell survival rate was measured by mitochondrial enzyme activity, which was detected by formazan formed from MTT (Denizot and Lang, 1986). As shown in Fig. 1, cell survival rate was reduced with 0.5% FBS in comparison with 10% FBS. Percentage of formazan formed in the survived cells by 200 μM H_2O_2 was about 55% with 0.5% FBS as compared to almost 100% with 10% FBS control. Data

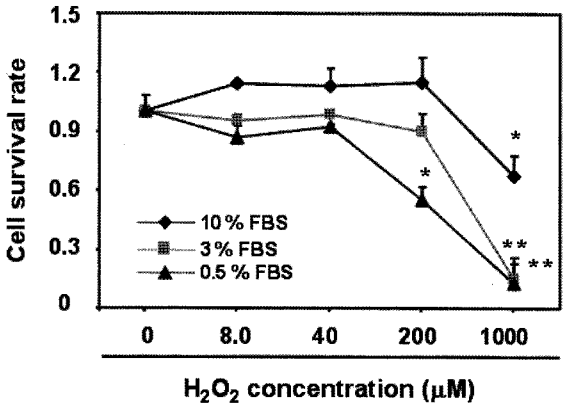

Fig. 1. H_2O_2-induced HeLa cervical tumor cell death was enhanced by serum deprivation. HeLa cells were plated at a density of 2×10^4 cells/ml and treated with various concentra-tion of H_2O_2 in the presence of 0.5, 3, and 10% FBS. Cell survival was measured with MTT assay as described in materials and methods. Cell survival rate was presented by the rate of absorbance (OD_{540nm}) at each group compared to control absorbance. Data in line graph represent mean± SED. *$p<0.05$; **$p<0.01$, significantly different from HeLa cell number at plating.

demonstrate that serum deprivation could affect intracellular sensitivity of tumor cells to extracellular stimulation.

Serum depreviation enhanced mitochondria-dependent apoptotic cell death

To investigate the effect of serum deprivation on HeLa cervical tumor cells, we examined serum deprivation-induced cellular changes by microscopic observation. As shown in Fig. 2A, cell morphology shrunk and some of cells were floated by the incubation with 0.5% FBS. Chromosomal condensation was also detected by DAPI staining in the culture with 0.5% FBS (Fig. 2B). When total cell number was determined by trypan blue exclusion assay, it was significantly reduced by 0.5% FBS (Fig. 2C). It demonstrates that HeLa cervical tumor cell death could be induced by serum deprivation.

In addition, serum deprivation-induced cellular changes were determined by flow cytometry analysis after staining cells with propidium iodide (PI). As shown in Fig. 3A, cell growth as judged by the percentage of S-phase was reduced by serum deprivation from 13.25% with 10% FBS to 5.51% with 0.5% FBS. Percentage of sub-G0/G1 apoptotic cell population was increased by serum deprivation from 5.7% with 10% FBS to 23.24% with 0.5% FBS (Fig. 3B). It was confirmed by the increase of caspase-3, and -9 activities (Fig. 4A). Serum deprivation-induced caspase-3

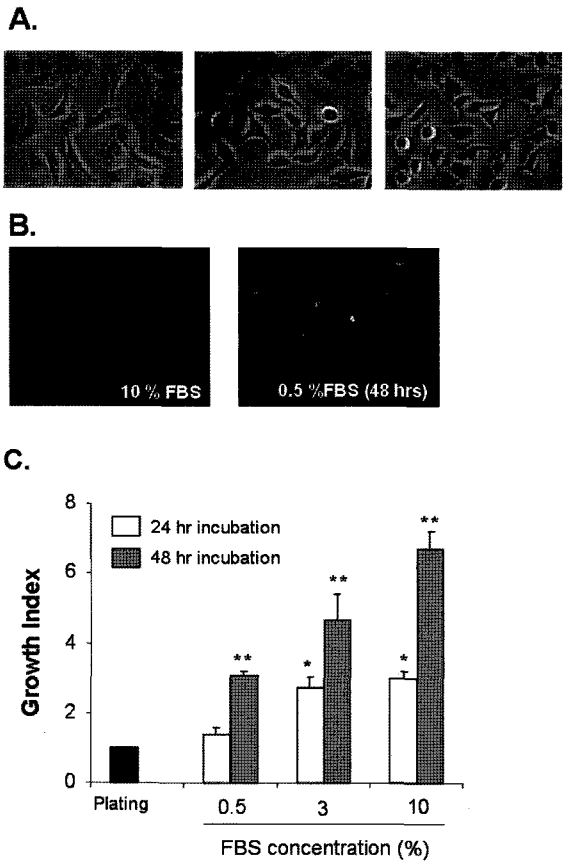

Fig. 2. Enhanced tumor cell death by serum deprivation. (A) 5× 10^3, 7.5×10^3, 1.0×10^4 HeLa cells were seeded in 96-well plate with 10, 3, 0.5% FBS, respectively and incubated for 48 hour. Cellular morphology was observed under the inverted microscope with a magnification of 200X. (B) Cells were fixed with 3% paraformaldehyde and stained with DAPI. Chromosomal changes were observed by fluorescence microscope with a magnification of 400X. (C) HeLa cells were plated at a density of 1×10^4 cells/ml in the presence of 0.5, 3, and 10% FBS. Cell number was counted under the inverted microscope at 24, and 48 hour after plating cells. Cell growth was presented as growth index by analyzing a relative cell number in 0.5, 3, and 10% FBS to plating.

activation was re-affirmed by the decrease of procaspase-3 protein amount (Fig. 4B). In addition, serum deprivation-induced caspase-9 activation was re-affirmed by the increase of cytochrome *c* release from mitochondria into cytosol (Fig. 4B). It suggests that serum deprivation-induced tumor cell death was mediated by mitochondria-dependent apoptotic cell death pathways.

Mitochondrial enzyme was activated by serum deprivation

We examine the effect of serum deprivation on the acti-

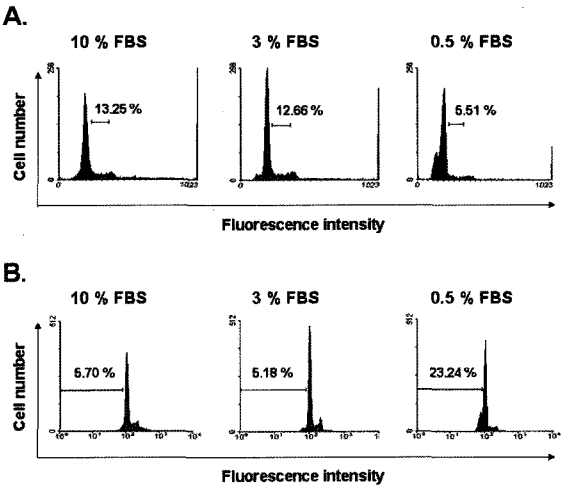

Fig. 3. Apoptotic cell analysis by flow cytometry. HeLa cells were incubated with 0.5, 3, and 10 % FBS for 24 hour. Cells were fixed and stained with 50 μg/ml propidium iodide and 25 μg/ml RNase. 10,000 cells were analyzed by flow cytometry. Percentage of cells was analyzed at S-phase (A) and sub-G0/G1 (B).

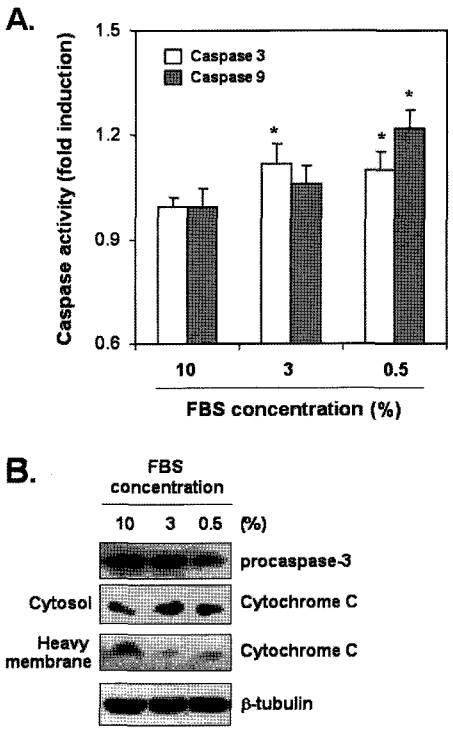

Fig. 4. Serum deprivation induced mitochondria-dependent apoptotic pathways. (A) HeLa cells were incubated with 0.5, 3, and 10% FBS for 24 hour. Caspase-3-like and caspase-9-like activity were assessed with the substrate N-Ac-DEVD-*p*-NA and N-Ac-LEHD-*p*-NA, respectively, by means of a colorimetric assay. Data in line graph represent mean±SED. **p*<0.05, signifi-cantly different from 10% FBS control. (B) HeLa cells were incubated with 0.5, 3, and 10 % FBS for 24 hour. Cell lysates were analyzed with SDS-PAGE. Molecular changes were detected by Western blot analysis.

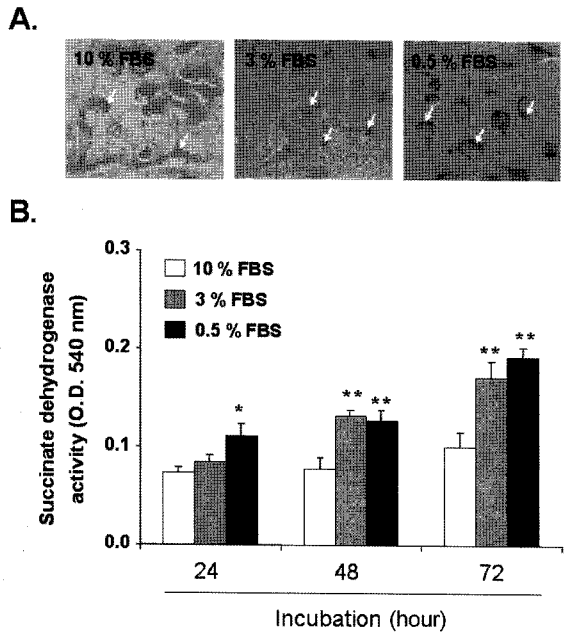

Fig. 5. The activation of mitochondrial enzyme by serum deprivation. (A) 5×10^3, 7.5×10^3, 1.0×10^4 HeLa cells were seeded in 96-well plate with 10, 3, 0.5% FBS, respectively and incubated for 24 hour. Formazan formed from MTT was shown as granule inside cells, which was observed under the inverted microscope with a magnification of 200X and indicated with arrows. (B) Cells were incubated with 0.5, 3, and 10% FBS for 24, 48, and 72 hour. Mitochondrial succinate dehydrogenase activities were measured by MTT assay. Formazan formed were dissolved in dimethylsulfoxide (DMSO). Optical density (OD) was read at 540 nm. Succinate dehydrogenase activity was presented by the absorbance (OD_{540nm}) for 5×10^3 cells at each group. Data in bar graph represent mean± SED. $^*p<0.05$; $^{**}p<0.01$, significantly different from 10% FBS control at each time point.

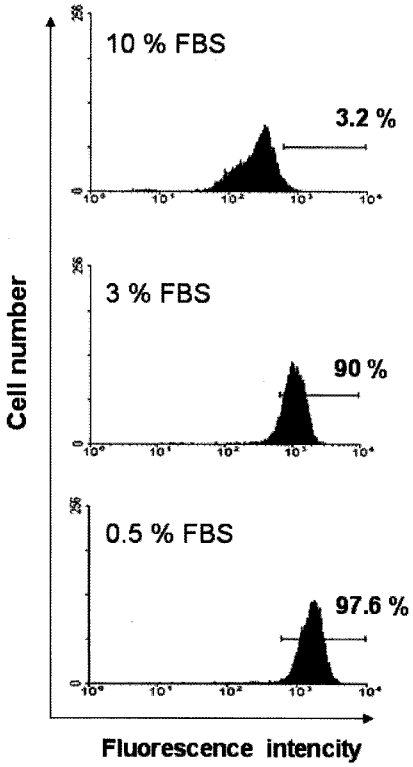

Fig. 6. Alteration of ROS level by serum deprivation. HeLa cells were incubated with 0.5, 3, and 10% FBS for 24 hour. ROS production was measured by the incubation with DCF-DA. 10,000 cells were analyzed by flow cytometry and ROS-positive cells were showed by mean fluorescence intensity (MFI) with more than 600. These data are representative of results obtained with three independent experiments.

vation of mitochondrial function. Since an insoluble formazan was formed by cleavage of the tetrazolium ring in MTT by dehydrogenase enzymes in mitochondria (Slater, et al., 1963; Denizot and Lang, 1986), mitochondrial succinate dehydrogenase activity in 5×10^3 cells might be correlated with formazan formed from MTT. As shown in Fig. 5A, formazan formation was increased by serum deprivation. Formazan amount in 5×10^3 cells was time-dependently increased from 24 hour- to 72 hour-incubation (Fig. 5B). It suggests that serum deprivation-induced apoptotic cell death could be mediated by mitochondrial enzyme activation.

Mitochondrial electron transport chain was activated by serum deprivation

Mitochondria are the main site to produce reactive oxygen species (ROS) at complex I, II, III of mitochondrial electron transport chain (Shin, *et al.*, 2007; Wang, *et al.*, 2007[a]; McLennan and Degli Esposti, 2000; Ott, *et al.*, 2007). Complex II (succinate dehydrogenase) contributes to the basal production of ROS in cells (McLennan and Degli Esposti, 2000). When cells were cultured in the presence of low percentage of FBS, ROS production was increased by 0.5 or 3 % FBS compared to 10% FBS (Fig. 6). When ROS-positive cells were analyzed by mean fluorescence intensity (MFI) with more than 600, percentage of them was 3.2% with 10% FBS and it was 97.6% with 0.5%. It demonstrates that mitochondrial electron transport chain was activated by serum deprivation.

To confirm that mitochondrial enzyme was activated in mitochondrial electron transport, cells were treated with rotenone, an inhibitor of mitochondrial electron transport (Chance, *et al.*, 1963). When mitochondrial succinate dehydrogenase activity in 5×10^3 cells was measured by formazan formed from MTT, it was significantly reduced by

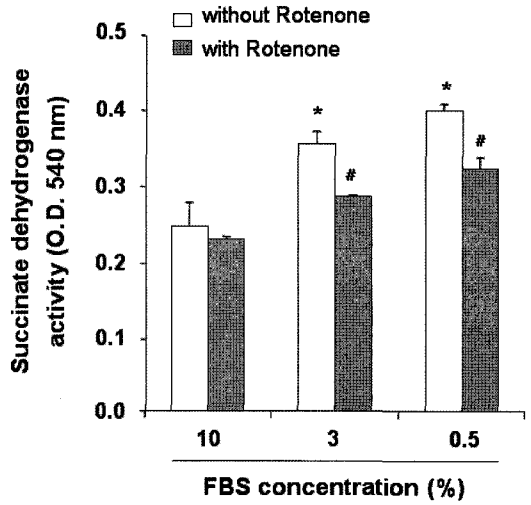

Fig. 7. Mitochondrial enzyme activity was reduced by rotenone, mitochondrial electron transport inhibitor. 5×10^3, 7.5×10^3, 1.0×10^4 HeLa cells were seeded in 96-well plate with 10 , 3, 0.5% FBS, respectively. HeLa cells were incubated with 0.5, 3, and 10% FBS in the presence or absence of 10 nM rotenone for 24 hours. Mitochondrial succinate dehydrogenase activities were measured by MTT assay. Formazan formed were dissolved in dimethylsulfoxide (DMSO). Optical density (OD) was read at 540 nm. Succinate dehydrogenase activity was presented by the absorbance (OD_{540nm}) for 5×10^3 cells at each group. Data in bar graph represent mean±SED. *$p<0.05$, significantly different from 10% FBS control. #$p<0.05$, significantly different from rotenone-untreated group at the same FBS concentration.

rotenone treatment with 0.5 and 3% FBS as compared to rotenone-untreated control (Fig. 7). The concentration of rotenone (10 nM) was low enough not to decrease succinate dehydrogenase activity with 10 % FBS. It suggests that serum deprivation could activate mitochondrial enzyme in mitochondrial electron transport and then it leads to ROS production and mitochondria-dependent apoptotic cell death.

DISCUSSION

Apoptosis can be induced by serum deprivation in many cell types (Bialik, *et al.*, 1999; Colombaioni, *et al.*, 2002[a]; Kazi and Dou, 2005; Zhu, *et al.*, 2006). Mitochondria play a key part in the regulation of apoptotic cell death (Susin, *et al.*, 1999[b]). Serum deprivation can activate the mitochondrial apoptotic pathway (Roucou, *et al.*, 2001). However, little has been known about whether the activation of mitochondrial enzyme and electron transport are involved in serum deprivation-induced apoptotic cell death. Our data show that mitochondrial succinate dehydrogenase and electron transports were activated by the prolonged serum deprivation in HeLa cervical tumor cells as judged by the formazan formed from MTT and ROS production, respectively. With 10 % FBS, the morphology of HeLa cells was normal, but with 0.5 or 3% FBS, apoptotic cell death was noted. These results suggest that serum deprivation could activate mitochondrial enzyme in mitochondrial electron transport and then it leads to mitochondria-dependent apoptotic cell death.

MTT assay was originally reported as a method to measure cell density (Denizot and Lang, 1986). Formazan was formed from MTT by succinate dehydrogenase, one of mitochondrial enzyme. Here, we found that the increase of cell number was retarded (Fig. 2C) and apoptotic cell death was elevated (Fig. 2B and Fig. 3) by the incubation with 0.5% FBS compared to that with 10% FBS, which was detected by DAPI or PI staining methods not by MTT assay. In contrast, we detected the increase of formazan by serum deprivation (Fig. 5), which was reduced by rotenone, a mitochondrial electron transport inhibitor (Fig. 7). It suggests that serum deprivation activate mitochondrial enzyme, which led to apoptotic cell death. These results also demonstrate that MTT assay is inapplicable to measure cell viability in serum deprivation although many reports used it to determine cytotoxicity by drugs in 10% FBS (Denizot and Lang, 1986; Moon and Lerner, 2003).

There are a few possibilities to explain the mechanism of action on serum deprivation-induced apoptosis. The first is the redistribution of Bcl-2 family proteins between cytosol and mitochondrial membrane. Apoptosis over 48 hour was concomitant with the increased cytochrome c in cytosol. However, both apoptosis and cytosolic cytochrome *c* fell dramatically at 72 hour. It is attributed to the elevation of whole cell Bcl-x(l) and redistribution of Bcl-x(l) protein from cytosol to mitochondrial membrane at 48 hour and 72 hour. Bax protein was also redistributed from the membrane to cytosol at 24 hour, and remained steady to 72 hour. (Zhang, *et al.*, 2000). Lovastatin protects mesenchymal stem cells from hypoxia/serum deprivation-induced apoptosis via PI3K/Akt and MEK/ERK1/2 pathways (Xu, *et al.*, 2008). p38 also plays a role in the inhibition of Bcl-2-induced apoptosis after serum starvation (Nelyudova, *et al.*, 2007). Serum deprivation-induced apoptosis could be mediated by the activation of p38 kinase, PI3K, and MEK/ERK followed by the redistribution of Bcl-2 family proteins.

The second possibility is ROS-mediated apoptosis by

serum deprivation. Oxidative stress from mitochondria play an important role in apoptosis (Ishii, *et al.*, 2007). Complex II (succinate dehydrogenase) contributes to the basal production of ROS in cells (McLennan and Degli Esposti, 2000). The inhibition of distal subunits of complex II increases normoxic ROS production (Guzy, *et al.*, 2008). Both the rapid loss of mitochondrial transmembrane permeability and the generation of ROS are due to the disrupted oxygen consumption on mitochondrial electron transport complexe II by activated caspase-3. (Ricci, *et al.*, 2003). In addition, ROS are generated at complex I including NADH dehydrogenase and complex III of mitochondrial respiratory chain (McLennan and Degli Esposti, 2000; Ott, *et al.*, 2007). Adaphostin, preclinical anticancer dihydroquinone derivative, enhanced the production of ROS by succinate-charged mitochondria (Le, et al., 2007). Our data also showed that serum deprivation increased succinate dehydrogenase activity, ROS production, chromosomal condensation, hypodiploid cell formation, and caspase-3 and -9 activity. Therefore, it suggests that serum deprivation induced apoptosis through ROS production by mitochondrial enzyme activation.

The last possibility is the role of serum deprivation-induced molecules such as ceramide and integrin $\beta 4$ on apoptotic cell death. The intracellular ceramide level increased by serum deprivation leads to calcium dysregulation and release of cytochrome *c* followed by caspase-3 activation (Colombaioni, *et al.*, 2002[b]). Deprivation of growth factors induced apoptosis and triggered senescence in human umbellical vascular endothelial cells (HUVECs) at which the level of integrin $\beta 4$ was increased markedly (Wang, *et al.*, 2007[b]). It suggests that ceramide and integrin $\beta 4$ might be a pivotal factor on the induction of apoptosis by serum deprivation.

In conclusion, even though we could not explain all phenomena on serum deprivation-induced apoptotic cell death pathways, the data demonstrate that serum deprivation increases mitochondrial enzyme activity and ROS production in mitochondrial electron transport, which results in mitochondria-dependent apoptotic cell death of HeLa cervical tumor cells. It is required to define further a detail mechanism of action on serum deprivation-induced apoptotic cell death pathways. This suggests for the first time that the activation of mitochondrial enzyme, such as succinate dehydrogenase could be an encouraging way to enhance the efficacy of anti-tumor agents on various tumor cells.

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