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Constitutive Activation of AKT Pathway Inhibits TNF-induced Apoptosis in Mitochondrial DNA-Deficient human myelogenous leukemia ML-1a

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Abstract

TNF plus protein synthesis inhibitor cycloheximide induced apoptosis in human myelogenous leukemia ML-1a but not in C19, respiration minus mitochondrial DNA deficient C19 cells, derived from ML-1a. To investigate how mitochondrial DNA depletion inhibits apoptosis, we investigated AKT. Both AKT and its phosphorylated form were observed only in C19, indicating that depletion of mtDNA increased protein and the active form of AKT. Treatment of C19 with LY294002, which inhibits PI-3 kinase and inhibits AKT, significantly increased apoptosis induction by TNF plus cycloheximide and eliminated phosphorylation of AKT. These results indicate that AKT activation was induced by the depletion of mtDNA and inhibited TNF-induced apoptosis.

Keywords

mitochondrial DNA; AKT; TNF

1. Introduction

Mitochondria are essential organelles that generate cellular energy (ATP) through oxidative phosphorylation, and this process is accomplished by a series of protein complexes and mitochondrial respiratory chains (MRC) encoded by nuclear DNA and mitochondrial DNA (mtDNA). Although ATP generation through oxidative phosphorylation is most efficient, it is not always required for cellular energy. Glycolysis can also generate ATP and provides compensatory mechanisms when oxidative phosphorylation becomes inefficient because of defects in the respiratory chain. Human mtDNA is remarkably small (16,569 bp) compared with nuclear DNA (approximately 10⁹ bp). MtDNA encodes only 13 polypeptides in the MRC, and the majority of mitochondrial respiratory proteins (at least 74) are encoded by nuclear DNA, translated in the cytoplasm, and then imported into mitochondria.

Approximately 70 years ago, Warburg pioneered research on mitochondrial respiratory alterations in the context of cancer and established the hypothesis [1]. In his publications, he hypothesized that a key event in carcinogenesis involved the development of an ‘injury’ to the respiratory machinery, resulting in compensatory increases in glycolytic ATP production.

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Several reports showed that mutations of mtDNA have been identified in various types of cancer including breast, colon, prostate, pancreatic, and others. Further, mutation of the D-loop region in cancer cells is substantially reported [2]. The D-loop region is responsible for controlling replication and transcription of mtDNA. Thus, D-loop alterations may interfere with promoter sequences and modify the binding affinities of the inducers and/or modulators of mtDNA transcription, changing the rate of transcription and replication of mtDNA [3]. It is likely that mtDNA mutation, deletion, or depletion induces the changes hypothesized by Warburg. The inheritance of mitochondrial haplotype U is associated with an approximately 2-fold increase in prostate cancer risk and a 2.5-fold increase in renal cancer risk in white, North American individuals [4], suggesting that mtDNA may affect cancer development. Interestingly, mutation of mtDNA in the D-loop region appears to be an indicator of poor prognosis in colorectal cancer patients and of resistance to fluorouracil-based adjuvant chemotherapy in stage III colon cancers [5].

MtDNA-deficient cells (p0) were established by long-term exposure to low concentrations of ethidium bromide [6]. Roles of mtDNA on several biological functions, including apoptosis, have been established by using p0 cells [7]. Very limited spectrum of cell type can be killed by TNF [8] and even in TNF-sensitive cells, it requires long incubation time (3 to 4 days) before we could observe death of the sensitive cell lines by TNF. We previously established the system to induce apoptosis in 90 min by TNF in the presence of protein synthesis inhibitor cycloheximide where apoptotic morphological change and DNA fragmentation is well-correlated [9]. Using this system, we showed that TNF and serum starvation could not induce apoptosis in respiration-deficient cells; whereas, they induced apoptosis in parental cells and cells reconstituted with normal mtDNA [10]. These results suggest that depletion of mtDNA leads cells to be resistant through activating certain anti-apoptosis pathway. Amuthan et al [11] demonstrated that mtDNA-depleted murine skeletal myoblast C2C12 cells showed invasive phenotypes and overexpression of the tumor-specific markers cathepsin L and TGF- β . This indicates that the loss of mtDNA may contribute to tumor progression and metastasis and this is probably due to inhibition of apoptosis. It is also known that mtDNA depletion can AKT in leukemia and lymphoma system [12]

In this study, a human myelogenous leukemia ML-1a, its mtDNA depleted cells C19, and the normal mitochondria reconstituted cybrid, P2 were used to investigate the role of non respiration mtDNA deficiency on apoptosis by TNF and cycloheximide. We reported that mtDNA deficient cells are resistant to TNF and cycloheximide [10]. Here, we report further investigation of the resistance and possible mechanisms to lead the resistance. The AKT activation in mtDNA deficient cells could be the main molecular key factor to inhibit TNF-induced apoptosis.

2. Materials and Methods

Materials

RPMI-1640 medium, gentamicin, FCS and Phosphate-buffered saline (PBS) were obtained from GIBCO (Grand Island, NY), cycloheximide, Tris, sodium dodecyl sulfate (SDS), LY294002, phenylmethanesulfonyl fluoride (PMSF), 2-mercaptoethanol, EDTA, ethidium bromide, uridine, glucose, pyruvate, polyethylene glycol 2000, Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO). Bacteria-derived recombinant human TNF, purified to homogeneity with a specific activity of 5×10^7 U/mg, was kindly provided by Genentech Inc. (South San Francisco, CA). Tris-EDTA Buffer Solution, pH7.6 (TE) was obtained from Fisher Scientific (Pittsburgh, PA). Anti-Phospho-Akt (Ser473) antibody, anti-phospho GSK3 β , anti-GSK3 β , anti-MCL-1 and anti- β -Actin antibody were obtained from Cell Signaling Technology, Inc. (Beverly, MA), anti-Akt1/2 (H-136) antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell lines

Myelogenous leukemia ML-1a cells were obtained from Dr. Ken Takeda (Showa University, Tokyo, Japan). ML-1a and P2 cells were grown in RPMI-1640 medium supplemented with 10% FCS and gentamicin (50 µg/ml) (essential medium). Clone 19 was grown in RPMI-1640 medium with 10% FCS and gentamicin (50 µg/ml) supplemented with 4.5 mg/ml glucose, 50 µg/ml uridine, and 100 µg/ml pyruvate (enriched medium). The cells were seeded at a density of 1×10^5 cells/ml in T-25 flasks (Falcon 3013; Becton Dickinson Labware, Lincoln Park, NJ) containing 10 ml of medium and grown at 37°C in an atmosphere of 95% air and 5% CO₂. Cell cultures were split every 3-5 d. MCFp0 were established from human breast cancer cell line MCF-7 by continuous treatment of 200 ng/ml ethidium bromide [13]. 143Bp0 mtDNA deficient cell line derived from human osteosarcoma 143B was kindly provided by Dr. Wei, Y. H. at National Yang-Ming University. MCFp0 and 143Bp0 are maintained in DMEM 5% FCS in the presence of 50 µg/ml uridine and 100 µg/ml pyruvate and MCF-7 and 143B are maintained in DMEM 5%FCS.

Establishment of respiration-deficient C19 and reconstituted clone P2

MtDNA deficient clone C19 from ML-1a and re-constituted clone P2 by the fusion of C19 with platelets was established as described [10].

DNA fragmentation assay

DNA fragmentation was assayed by the modified method as described elsewhere [9]. In brief, ML-1a cells were prelabeled with [³H]TdR by incubating 2×10^5 cells/ml in essential medium with 0.5 µCi/ml [³H]TdR at 37 ° C for 4-16 h. The cells were washed three times and resuspended in RPMI-1640 medium and plated in 96-well plates (4×10^4 cells/well, total volume 200 µl) with or without the test samples; 1 µg/ml of cycloheximide was added to increase the sensitivity to 1 nM TNF with or without 40 µM or the indicated concentration of LY294002. After incubation for the indicated time, they were lysed by the addition of 50 µl of detergent buffer (10 mM Tris-HCl pH 8.0 containing 5 mM EDTA and 2.5% Triton X-100) and incubated an additional 15 min at 4 ° C. High-speed centrifugation was performed in an Eppendorf microcentrifuge at 12,000 g for 1 min. The radioactivity in the supernatant represents DNA release into cells due to DNA fragmentation. For the total count, the cells were lysed by the addition of 20 µl of 20% SDS. The percent DNA release was calculated as follows: %DNA fragmentation = (cpm in test sample supernatant / total cpm) × 100 %DNA fragmentation is well co-related with % of the cells with apoptotic morphology [9]. All results were determined in triplicate and expressed as mean ±standard error.

Western blot analysis

After incubation of $2-3 \times 10^6$ cells under certain conditions, each cell was harvested by centrifugation at 188 g for 5 min. After washing with PBS, the pellet cells were suspended in 50 µl lysis buffer (1% Triton X-100, 2 mM PMSF, 0.02% 2-mercaptoethanol in 50 mM Tris-HCl, pH 7.2) and lysed on ice for 10 min. The homogenate was subjected to centrifugation at 12,000 g for 10 min, and then the supernatant was collected to obtain a total cell extract. Protein concentrations were determined with the Bio-Rad Protein assay (Bio-Rad Laboratories). Samples of the total cell extracts containing 100 µg proteins were subjected to electrophoresis using 10% polyacrylamide slab gels in the presence of 0.1% SDS. After electrophoresis, the proteins were transferred electrophoretically to nitrocellulose membrane (Bio-Rad Laboratories). Immunodetection was carried out by WesternBreeze® Chemiluminescent Western Blot Immunodetection Kit (Invitrogen Corporation, Carlsbad, CA). The nonspecific binding sites on the membrane were first blocked with 1% (w/v) dried milk in the blocking solution for 1 h at room temperature. The membrane was incubated with specific primary antibodies diluted 1 : 1000, respectively in 1% (w/v) dried milk in primary antibody solution

for 2 h at room temperature or over night at 4 ° C. After four washes with washing solution, the membrane was incubated with secondary antibody solution (goat anti-rabbit IgG alkaline phosphatase-conjugate) with 1% (w/v) dried milk for 30 min at room temperature. The membrane was then washed four times with washing solution, stained with Chemiluminescent substrate with substrate enhancer according to supplier's instructions, exposed to X-ray film or scanned by Gel Doc System (Bio-Rad Laboratories).

3. Results

Inhibition of apoptosis induced by TNF plus cycloheximide

We previously established a mitochondrial respiration-deficient clone (C19) from human myelogenous leukemia ML-1a and a reconstituted clone (P2) through the fusion of C19 cells with platelets from a healthy donor. C19 did not consume oxygen and could not survive in the absence of pyruvate and uridine [10]. As shown in Figure 1, we confirmed the following observations, as previously reported [10]. 1) TNF with cycloheximide induced apoptosis in ML-1a cells in 90 min. 2) Apoptosis induced by TNF plus cycloheximide was almost eliminated in C19 cells. 3) Apoptosis induced by TNF plus cycloheximide was recovered in the reconstituted clone P2. 4) TNF or cycloheximide alone had no or small effect on ML-1a, C19 and P2.

AKT pathways in mtDNA-deficient C19 cells are constitutively increased

AKT activation is known to inhibit TNF-induced cell death [14]. To investigate possible roles of AKT in the resistance of C19 cells to TNF-induced apoptosis, we first tested AKT in ML-1a, C19, and reconstituted P2 cells by Western blotting. As shown in Figure 2A, we could detect a large amount of AKT protein in C19 cells, but not in ML-1a and P2. Because activation of AKT is dependent on phosphorylation of molecules at definite sites, we can determine the active form of AKT by using antibodies specific to Phospho-AKT. We detected Phospho-AKT in C19 cells, but not in ML-1a and P2 (Figure 2A). Therefore, mtDNA depletion caused increases in AKT and its active form, and reconstitution of mtDNA to mtDNA-depleted cells reduced the expression of AKT and phosphorylated AKT. To investigate whether AKT activation by mtDNA reduction is observed only in ML-1a system or not, we used different mtDNA deficient cell lines from several cell lines as follows. We established respiration-deficient cell lines by low concentration of ethidium bromide for a couple of months; MCFp0 from human breast cancer MCF-7. We also used 143Bp0 established from human osteosarcoma 143B. Faint band of phospho-AKT was detected in parental MCF-7 and 143B and greatly increased in MCFp0 and 143p0 (Figure 2B). These results suggest that reduction of mtDNA content activates AKT pathway.

Downstream of AKT pathways in mtDNA-deficient cells are upregulated

AKT activation is followed by the phosphorylation of certain molecules leading to the shift of enhancement of glycolysis and/or anti-apoptotic pathways [15]. Glycogen synthase kinase 3 β (GSK-3 β) is a direct substrate for AKT and the activation of GSK-3 β increases glycolysis and reduce oxidative phosphorylation. As shown in Figure 2A, phospho-GSK3 β was only observed in C19 cells but not in ML-1a and P2. This result suggests that phosphorylation of GSK3 β was induced in mtDNA depletion in ML-1a system. Enhancement of phospho-GSK3 β band was observed in 143Bp0 as compared with the parental cell line 143B (Figure 2B) suggesting that GSK3 β was activated in 143Bp0 cells. However, significant enhancement of the band representing phospho GSK β by mtDNA depletion was not observed in MCFp0 (Figure 2B).

MCL-1 is an anti-apoptotic/differentiation-related bcl-2 family protein and considered one of the causes for acute myelogenous leukemia [16]. MCL-1 is an anti-apoptotic protein and its expression is regulated by AKT pathway [17]. As shown in Figure 2A, we could detect MCL-1

in C19 but not in ML-1a and P2. Enhancement of MCL-1 band was observed in 143B p0 compared with 143B and MCFp0 compared with NCF-7 respectively (Figure 2B). These results strongly suggest that the downstream event of AKT activation is also upregulated by depletion of mtDNA. We did not observe enhancement of the expression of anti-apoptotic Bcl-2 and Bcl-xl and decrease of proapoptotic Bax in mtDNA-deficient cells (Data not shown).

Constitutive active AKT in C19 is responsible for inhibition of TNF-induced apoptosis

AKT activation is known to be the downstream target of the PI-3 kinase. We investigated the effect of LY294002, an inhibitor of PI-3 kinase, on TNF-induced apoptosis in C19 cells. We incubated C19 cells with TNF plus cycloheximide in the presence or absence of LY294002 and incubated for 1.5, 3, 5 and 7 hours. LY294002 treatment for seven hours significantly increased apoptosis induction in C19 cells with TNF plus cycloheximide from $21.8 \pm 0.9\%$ to $66.8 \pm 2.3\%$. (p value < 0.01 student t test) (Figure 3). The effect of LY294002 was not obvious if the incubation time is less than five hours. LY294002 alone had no effect, and TNF plus cycloheximide slightly increased apoptosis induction after seven hours incubation. We next investigated the dose-dependent effect of LY294002 on apoptosis induced by TNF plus cycloheximide (Figure 4A). As expected, TNF plus cycloheximide induced slight, but significant, induction of apoptosis in C19 cells from $16.4 \pm 0.6\%$ to $25.1 \pm 0.4\%$ (p value < 0.01 student t test), and 40 μM of LY294002 greatly increased apoptosis in C19 cells incubated with TNF plus cycloheximide from $16.4 \pm 0.6\%$ to $41.1 \pm 1.0\%$ (p value < 0.01 student t test). Twenty μM of LY294002 slightly increased apoptosis induced by TNF plus cycloheximide. In contrast, LY29002 alone could not induce apoptosis. At the same time, we investigated the effect of LY294002 on phosphorylated AKT by Western blotting. As shown in Figure 4B, 20 μM of LY294002 reduced phosphorylation of AKT, and 40 and 80 μM of LY294002 greatly inhibited phosphorylation.

We next tested time-dependent effect of LY294002 on AKT pathway in the seven-hour culture. We investigated the effect of LY294002 on AKT, phosphorylated AKT, GSK-3 β , phosphorylated GSK-3 β , MCL-1 and β -actin (Figure 5). LY294002 did not affect total AKT. LY294002 inhibited AKT activation when C19 cells were treated with LY294002 for one hour and the effect continued at least up to seven hours. In contrast, the effect of LY294002 on phosphorylated GSK-3 β and MCL-1 expression was only observed seven hours after LY294002 was added (Figure 5). These results suggest that, although the activation of AKT was inhibited in an hour, approximately seven hours were needed before GSK-3 β phosphorylation and MCL-1, an anti-apoptotic molecule and the downstream of AKT, were inhibited by LY294002.

4. Discussion

In this study, we examined the roles of constitutive active AKT on the inhibition of TNF-induced apoptosis by depleting mtDNA and mitochondrial respiratory function. We previously demonstrated that TNF in the presence of cycloheximide induced apoptosis in ML-1a parental cells in 90 minutes [9], but the apoptosis was blocked in mtDNA-deficient C19 cells [10]. To investigate the mechanism involved in apoptosis inhibition by mtDNA depletion, we focused on AKT activation because we observed constitutive expression of AKT and phosphorylated AKT in mtDNA-deficient C19 cells. Interestingly, these molecules were not observed in parental ML-1a cell lines and disappeared in the reconstituted clone P2, indicating that mtDNA negatively regulates the expression of AKT and phosphorylated AKT directly or indirectly. LY294002, an inhibitor of the PI-3 kinase, reduced AKT activation within one hour and took seven hours for C19 cells to recover sensitivity to TNF plus cycloheximide, which induces apoptosis in these cells.

There are several possible mechanisms downstream of AKT activation to inhibit apoptosis in C19 cells. Phosphorylated AKT is a well established survival factor preventing, in part, the release of cytochrome c from mitochondria [18]. Phosphorylated AKT also inactivates the pro-apoptotic factors BAD and procaspase-9 [19]. Inhibition of caspase-9 activation may not be the cause because of the following reasons. Caspase-9 is involved after cytochrome c release, and we could detect cytochrome c release in ML-1a and cybrids, but not in C19 cells [20]. This result strongly suggests that mechanisms upstream of cytochrome c release are inhibited in C19 cells. Because it took seven hours before AKT phosphorylation is reduced by LY294002 to recover sensitivity to TNF plus cycloheximide in C19, LY294002 might induce several mechanistic sequences before anti-apoptotic mechanisms shut down. MCL-1 is a anti-apoptotic protein and its expression is regulated by AKT pathway [17]. Regarding upstream event of AKT activation followed by mtDNA depletion, Pelicano et al showed the possible contribution of increased NADH induced by mtDNA deficiency may inactivate PTEN leading to the activation of AKT [12].

AKT activation has been considered a possible cause of cancer progression and initiation [21]. We recently observed that androgen ablation therapy depleted mtDNA, leading to a more progressed, androgen-independent phenotype in prostate cancer [22]. Additionally, mtDNA mutation in the D-loop region, which may reduce mtDNA replication [3], is very common in several cancers [2]. Therefore, mtDNA depletion, possibly caused by mtDNA mutation in the D-loop region or some other causes, may lead to activation of AKT, aiding cancer progression and initiation.

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Figure 1.

Induction of DNA fragmentation by TNF and cycloheximide in ML-1a, C19, and P2. [^3H]TdR prelabeled cells incubated with 1 nM TNF and 1 $\mu\text{g}/\text{ml}$ cycloheximide for 90 min were tested for DNA fragmentation.

Figure 2.

AKT and downstream of AKT in ML-1a, C19, P2 (A), MCF-7, MCFp0, 143B and 143Bp0 (B). $2-3 \times 10^6$ cells were lysed and subjected to Western blotting analysis using anti-Akt1/2, anti-Phospho-Akt (Ser473), anti-phospho GSK3 β , anti-MCL-1 and anti- β -Actin.

Figure 3.

The effects of LY294002 on apoptosis induction with TNF plus cycloheximide in C19 cells. Forty μ M LY294003 was added to [3 H]TdR prelabeled C19 cells with or without 1 nM TNF plus 1 μ g/ml cycloheximide and tested for DNA fragmentation at the indicated times.

Figure 4.

Dose-dependent effects of LY294002 on apoptosis induction with TNF plus cycloheximide (A) and AKT activation (B). (A) Indicated amount of LY294002 was added to [3 H]TdR prelabeled C19 cells for 7 hours with or without 1 nM TNF plus 1 μ g/ml cycloheximide and tested for DNA fragmentation. (B) C19 cells were incubated with the indicated amount of LY294002 for 1 hour, lysed, and subjected to Western blotting analysis using anti-Phospho-Akt (Ser473) and anti-Akt.

Figure 5.

The effects of LY294002 on AKT pathways in C19 cells. C19 cells were incubated in the presence of 40 μ M LY294002 for the indicated times, lysed, and subjected to Western blotting analysis using anti-Akt1/2, anti-Phospho-Akt (Ser473), anti-GSK3 β , anti-phospho GSK3 β , anti-MCL-1 and anti- β -Actin.