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Suppression of oxidative phosphorylation in mouse embryonic fibroblast cells deficient in apurinic/apyrimidinic endonuclease

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ABSTRACT

The mammalian apurinic/apyrimidinic (AP) endonuclease 1 (APE1) is an essential DNA repair/gene regulatory protein. Decrease of APE1 in cells by inducible shRNA knockdown or by conditional gene knockout caused apoptosis. Here we succeeded in establishing a unique mouse embryonic fibroblast (MEF) line expressing APE1 at a level far lower than those achieved with shRNA knockdown. The cells, named MEF^{la} ($MEF^{lowAPE1}$), were hypersensitive to methyl methanesulfonate (MMS), and showed little activity for repairing AP-sites and MMS induced DNA damage. While these results were consistent with the essential role of APE1 in repair of AP sites, the MEF^{la} cells grew normally and the basal activation of poly(ADP-ribose) polymerases in MEF^{la} was lower than that in the wild-type MEF (MEF^{wt}), indicating the low DNA damage stress in MEF^{la} under the normal growth condition. Oxidative phosphorylation activity in MEF^{la} was lower than in MEF^{wt}, while the glycolysis rates in MEF^{la} were higher than in MEF^{wt}. In addition, we observed decreased intracellular oxidative stress in MEF^{la}. These results suggest that cells with low APE1 reversibly suppress mitochondrial respiration and thereby reduce DNA damage stress and increases the cell viability.

1. Introduction

Aerobic energy generation in eukaryotic cells takes place in mitochondria through oxidative phosphorylation (OXPHOS). While glycolysis is less efficient than OXPHOS in generating ATP, cancer cells often need to survive under hypoxic conditions due to undeveloped vasculature. This hypoxia compels cancer cells to utilize glycolysis to much larger extent than normal cells to generate ATP [1]. A recent finding that rates of random mutations in mitochondrial DNA were lower in cancer cells than in normal cells [2] is consistent with the fact that reactive oxygen species (ROS) are generated due to leaked electrons during OXPHOS [3]. Together these studies indicate that a consequence of active mitochondrial respiration is a higher risk of DNA damage.

Endogenous DNA damage includes base damage, apurinic/apyrimidinic (AP) sites and DNA single-strand breaks (SSBs) which are generated at rates more than 10,000/cell/day in normal conditions [4,5]. In addition, recent studies have demonstrated highly active cytosine methylation and demethylation that generate AP sites in the genome [6,7]. These DNA lesions are primarily repaired via the DNA base excision repair (BER) process in which AP-endonucleases play a pivotal role in generating 3′-OH termini required for the DNA repair synthesis by DNA polymerases [8,9]. Both *Escherichia coli* and yeast cells harbor two functional AP endonuclease genes, and double mutants of these genes are still viable, because the repair of AP sites can be carried out by the backup activities provided by the DNA nucleotide excision repair (NER) [10,11]. In contrast, the mammalian AP endonuclease (APE1) is essential for cellular viability [12–16], underscoring the unique requirement of APE1 for repairing endogenously generated DNA damage. However, while an earlier study reported apoptosis in cells by APE1 down-regulation with an inducible shRNA system [16],

Abbreviations: AP site, apurinic/apyrimidinic site; APE, AP endonuclease; hAPE1 or mApe1, human or mouse AP endonuclease 1; ARP, aldehyde reactive probe; CRISPR, clustered regularly interspaced short palindromic repeats; EDD, endogenous DNA damage; EDDR, EDD repair; kd, knockdown; ko, knockout; MMS, methyl methanesulfonate; NER, DNA nucleotide excision repair; NEIL, endonuclease VIII-like; OXPHOS, oxidative phosphorylation; PARP, Poly (ADP-ribose) polymerase; PNKP, polynucleotide kinase phosphatase; ROS, reactive oxygen species; SSB, DNA single-strand breaks; UK, University of Kentucky; wt, wild-type; XRCC1, X-ray cross complementation group 1.

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cell lines with stable APE1 down-regulation have been successfully established in multiple laboratories [17–20]. The difference may be explained by a cellular adaptation to the low APE1 levels.

A possible mechanism for the cellular adaptation is an increase of repair activities independent of APE1. In principle, AP sites and SSBs in mammalian cells can be repaired without involving APE1 by SSB repair pathway in which PNKP (polynucleotide kinase/3′-phosphatase) plays the central role [9,21–24]. The lack of APE1-deficient cell lines or a mouse model hinders further investigations for unraveling the roles of APE1 and APE1-independent BER in protecting cells from endogenous DNA damage.

In this study, we established and examined unique mouse embryonic fibroblasts (MEF) that express APE1 at a level near threshold and far lower than those achieved with shRNA knockdown. Yet the APE1 deficient cells grew normally. Further investigation led us to conclude that intracellular levels of APE1 influence mitochondrial respiration activities, i.e., oxidative phosphorylation, thus reducing the cellular oxidative stress. These results indicate unexpected resilience of cells, which may have significant implications for cancer therapeutics that target APE1.

2. Materials and methods

2.1. Cell culture

The human colon carcinoma cell line HCT116 [20,25,26] and JHU28, a human squamous cell carcinoma line from head and neck cancer [27] kindly provided by Dr. Walvekar, were grown in DMEM High glucose (Hyclone) supplemented with 10% FBS (Gemini Bio-products), 1% L-glutamine, and 1% streptomycin/penicillin (Hyclone). A human APE1 shRNA adenovirus vector was a generous gift from Dr. Crowe [19]. The APE1 shRNA in the retrovirus vector pSIREN-RetroQ (Clontech) were introduced into HCT116 and JHU28 along with vector only controls, and then maintained in growth medium containing 1 μg/mL puromycin (Invivogen).

2.2. Isolation of mouse embryonic fibroblasts

Generation of APE1 transgenic mice and isolation of MEF from 9.5E embryos were described previously [13,15]. All animal procedures were carried out at University of Texas Medical Branch (UTMB) in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by UTMB Animal Care and Use Committee (#00-01-007). Briefly, mating pairs of mouse Ape1 gene-heterozygous and hAPE1 transgene positive ($mApe1^{+/-}$ $hAPE1^{Tg}$) C57BL/6 mice were used to generate embryos. The MEF line was screened by PCR [15] for the genotype of mouse Ape1 gene-homozygous and hAPE1 transgene positive ($mApe1^{+/-}$ $hAPE1^{Tg}$). Analyses of mApe1 and hAPE1 transcripts were carried out with reverse-transcriptase PCR using total RNA from MEFs (RNeasy Micro Kit, Qiagen) and PCR primers specific to the mouse and human RNA. Primers (Integrated DNA Technologies) for human APE1 mRNA were 5′-GCTTCGAGCCTGGATTAAGA-3′ and 5′-TTGGTCTCTTGAAGGCACAGT-3′, and those for mouse Apex mRNA were 5′-CCATTCTTTGTGCCGTGAG-3′ and 5′-GAGCACCGAAGCAGTGTTTA-3′. The 18s rRNA transcripts were used as an internal control (primers: 5′-GCAATTATTCCCCATGAACG-3′ and 5′-GGGACTTAATCAACGCAAGC-3′). The MEFs were transformed with SV40 T-antigen [15], and stably transfected with pFRT/lacZeo (Invitrogen). The MEF^{wt} ($mApe1^{+/+}$; $hAPE1^{Tg}$), was also isolated from a sibling, and used as a control. All cells were grown in DMEM (high glucose) supplemented with 10% FBS, 1% L-glutamine and 1% streptomycin/penicillin. The wild-type (wt) human APE1 (hAPE1) and mouse Ape1 (mAPe1) cDNA [20] were cloned into the pcDNA5.1FRT/TO vector (Invitrogen), and isogenic MEF^{la} derivatives expressing hAPE1 or mApe1 stably were established via Flp/FRT site-specific recombination [28]. Cells expressing hAPE1 and mApe1 genes were named MEF^{la}/hAPE1 and MEF^{la}/mApe1, respectively. Control cells with the empty vector were named MEF^{la}/vec. Supporting Fig. S1A provides a flowchart for construction of the MEF lines.

2.3. Immunoblot analysis

Cells were washed in PBS twice and lysed in RIPA lysis buffer (20 mM Tris-Cl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton-X, 0.1% SDS, 1 mM DTT), containing proteinase inhibitor cocktail (Roche). The total fractions of cell extracts were run in 10% SDS/PAGE, transferred onto PVDF membranes (Bio-Rad), which were blocked in 5% nonfat milk (Bio-Rad). The membranes were then blotted with appropriate primary and secondary antibodies listed below, and developed with chemiluminescent substrates (DURA or FEMTO, Pierce). Intensities of unsaturated (non-overexposed) signals were analyzed using ChemiDoc (Bio-Rd) and ImageJ software (NIH).

2.4. Cytotoxicity assays

Cells were plated (60 mm dishes at 2000 cells/dish), incubated for overnight and then treated with methyl methanesulfonate (MMS, Sigma) for 1 h at 37 °C. After removing MMS, cells were grown without MMS for 7–9 days or until colonies grew 1 mm in size, at which point the colonies were stained with crystal violet (Fisher) and counted to calculate survival fractions by dividing numbers of colonies of MMS-treated dishes (S) with those of untreated dishes (S0). A colorimetric assay (WST-8, Dojindo) to measure cellular NADPH dehydrogenase activity was also used to assay MMS cytotoxicity. Cells (5000/well) were incubated for 3 h on 96 well plates, treated with MMS for 1 h, washed and incubated for 24 h before the dye-formation assay at 450 nm (Emax, Molecular Device).

2.5. AP-endonuclease activity assay

Intracellular AP-endonuclease activity was examined using an oligonucleotide substrate as previously reported [29] with following modifications. A 32-mer 5′-Cy5-labeled oligonucleotide containing a single tetrahydrofuran (THF, 5′-AGGCCAATGATCGGTAT/TET/AAGTCGCGGGATAA-3′) and its reverse-complementary oligo were synthesized by Integrated DNA technologies (Coralville, IW). The duplex substrate DNA was incubated with 10 ng nuclear extracts [30] of MEFs at 37 °C for 8 min The DNA was then subsequently separated in denaturing 20% polyacrylamide gel electrophoresis (8% urea, Tris-borate buffer) at 65 °C. The substrate and cleaved products were analyzed with GeneStorm (General Electric).

2.5.1. Analysis of basal levels of cellular DNA damage with Comet assay

Comet assay was carried out according to vendor's manual (Trevigen, Inc.). Briefly, cells were plated 1 day before the assay, and resuspended in PBS at 1×10^5/mL. Cells were then embedded in low melting point agarose gel and lysed. The cells were immersed in alkaline solution to denature DNA, and gel electrophoresis was performed at 300 mA for 30 min After washing in H_2O, gels containing the cells were treated in 70% ethanol, dried for 15 min, and were stained in SYBR Gold (Invitrogen). Gels were then washed in H_2O and dried. Three independent experiments were carried out, and the comet tails of 30 individual cells were analyzed for each assay.