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AP endonuclease knockdown enhances methyl methanesulfonate hypersensitivity of DNA polymerase β knockout mouse embryonic fibroblasts

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ABSTRACT

Apurinic/apyrimidinic (AP) endonuclease (Apex) is required for base excision repair (BER), which is the major mechanism of repair for small DNA lesions such as alkylated bases. Apex incises the DNA strand at an AP site to leave 3′-OH and 5′-deoxyribose phosphate (5′-dRp) termini. DNA polymerase β (PolB) plays a dominant role in single nucleotide (Sn-) BER by incorporating a nucleotide and removing 5′-dRp. Methyl methanesulfonate (MMS)-induced damage is repaired by Sn-BER, and thus mouse embryonic fibroblasts (MEFs) deficient in PolB show significantly increased sensitivity to MMS. However, the survival curve for PolB-knockout MEFs (PolBKOs) has a shoulder, and increased sensitivity is only apparent at relatively high MMS concentrations. In this study, we prepared Apex-knockdown/PolB-knockout MEFs (AKDBKOs) to examine whether BER is related to the apparent resistance of PolBKOs at low MMS concentrations. The viability of PolBKOs immediately after MMS treatment was significantly lower than that of wild-type MEFs, but there was essentially no effect of Apex-knockdown on cell viability in the presence or absence of PolB. In contrast, relative counts of MEFs after repair were decreased by Apex knockdown. Parental PolBKOs showed especially high sensitivity at >1.5 mM MMS, suggesting that PolBKOs have another repair mechanism in addition to PolB-dependent Sn-BER, and that the back-up mechanism is unable to repair damage induced by high MMS concentrations. Interestingly, AKDBKOs were hypersensitive to MMS in a relative cell growth assay, suggesting that MMS-induced damage in PolB-knockout MEFs is repaired by Apex-dependent repair mechanisms, presumably including long-patch BER.

KEYWORDS: Apex, BER, Polβ, MEF, MMS, mouse

INTRODUCTION

Various types of DNA damage are generated under physiological conditions in mammalian cells. This damage includes apurinic/apyrimidinic (AP) sites and alkylated bases, which are repaired primarily via base excision repair (BER) pathways. In BER, an AP site is formed after base removal by a glycosylase. AP endonuclease then incises the DNA strand at the AP site to leave 3′-OH and 5′-deoxyribose phosphate (5′-dRP) termini. BER occurs through two downstream pathways: single nucleotide (Sn-) BER and long patch (Lp-) BER. In the Sn-BER pathway, the product is passed to DNA polymerase β (PolB), as proposed in the Passing the Baton model [1], and PolB inserts the correct nucleotide at the 3′-OH terminus and

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removes the 5′-dRP motif. In the Lp-BER pathway, two to eight nucleotides are incorporated by DNA polymerase δ/ε in a process that is dependent on proliferating cell nuclear antigen (Pcna). Thus, PolB plays a dominant role in Sn-BER [2], while AP endonuclease is essential in both BER pathways.

APE1 and Apex are major AP endonucleases in human and mouse cells [3, 4, 5] and have mostly similar biochemical and biophysical properties [6]. APE1 has a role in cellular protection against DNA damaging agents [7], and Apex-knockout mice show embryonic lethality [8]. Since APE1- or Apex-knockout cell lines are not available, the significance of these enzymes in cellular protection has been studied using APE1- or Apex-knockdown cells.

Human and mouse PolB both have DNA polymerase and dRP lyase activities. Sobol *et al.* demonstrated that mouse embryonic fibroblasts (MEFs) deficient in PolB are hypersensitive to >1 mM methyl methanesulfonate (MMS), with an apparent resistant shoulder below 0.5 mM [9]. PolB deficiency results in an increased apoptotic cell fraction and chromosomal aberrations after MMS treatment [10]. MMS hypersensitivity can be reversed by the dRP lyase domain of PolB [11]. These results suggest that this hypersensitivity is mainly caused by Sn-BER deficiency. A contribution of PolB-independent repair mechanisms is also likely because of the increased sensitivity of PolB-knockout MEFs at relatively high MMS concentrations [9].

Because DNA polymerase λ (PolL) belongs to the same family X and has similarities in activity and structure to PolB, PolL may play a backup role in the absence of PolB [12, 13]. The MMS sensitivity of chicken DT40 cells lacking both PolB and PolL did not differ from that of cells lacking only PolB [14]. More recently, MEF lacking both PolB and PolL was established. Although the sensitivity of PolL-deficient MEF to MMS did not significantly differ from that of WT cells, the double knockout MEF showed higher sensitivity to MMS than the MEF deficient in either of the polymerases [15]. Thus, in MEF, PolB and PolL seem to participate in the repair of common MMS lesions. PolB-deficient MEF shows resistance to low doses (0.5 mM) of MMS. Since PolB/PolL double knockout MEFs still showed an apparent resistance to MMS at low concentrations, a different system might contribute to the tolerance of a limited number of MMS lesions, which might be independent of backup by PolL. To obtain further information about the nature of the resistance at low MMS concentrations, we investigated the effect of Apex knockdown on the MMS sensitivity of PolB-knockout MEFs.

MATERIALS AND METHODS

Cell lines

Wild-type (Mβ16tsA) and PolB-knockout (Mβ19tsA) MEFs were generous gifts from Dr Masahiko Miura (Tokyo Medical and Dental University, Tokyo, Japan). These cell lines were cultured in Eagle's MEM "Nissui" 1 (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (Thermo Scientific, Waltham, MA), 1% MEM non-essential amino acids solution (Gibco BRL, Carlsbad, CA) and 1% sodium pyruvate solution (Gibco BRL) at 37°C in 5% CO_2.

Apex knockdown

A knockdown target sequence was selected using siRNA Wizard software (InvivoGen, San Diego, CA) based on the mouse Apex nucleotide sequence (NCBI: NM_009687.1). The sequence was located in the AP endonuclease domain of Apex. A short hairpin oligonucleotide (5′-ACC TCG GAT CTC AAT GTG GCT CAT GAT CAA GAG TCA TGA GCC ACA TTG AGA TCC TT) including the knockdown sequence (Sigma–Aldrich, St Louis, MO) was inserted into a psiRNA-hH1GFPzeoG2 shRNA expression vector (InvivoGen). The plasmid was transfected into *Escherichia coli* JM109 using a Cell-Porator™ (Gibco BRL), amplified in LB medium containing 25 μg/ml Zeocin (InvivoGen), and purified using a QIAprep spin Miniprep Kit (Qiagen, Hilden, Germany). The nucleotide sequence was confirmed by EQ8000 (Beckman Coulter, Brea, CA). The plasmid was introduced into MEFs using HilyMax (Dojindo, Kumamoto, Japan). Transfected cells were selected by renewing the medium containing 500 μg/ml Zeocin every three or four days.

Western blot analysis

After cloning each knockdown cell, exponentially growing cells were harvested, washed in cold PBS(-), and lysed in SDS gel-loading buffer (125 mM Tris-HCl, pH 6.8, 10% 2-mercaptoethanol, 4% SDS, 10% sucrose). After electrophoresis on a 12% SDS-polyacrylamide gel, proteins were transferred onto Immobilon-P Transfer Membranes (Millipore, Billerica, MA). After blocking with 5% non-fat milk in TPBS [0.1% Tween 20 in PBS(-)], the membranes were incubated with rabbit polyclonal anti-APE1 antibody (ab82, Abcam, Cambridge, MA). To normalize the amount of Apex, monoclonal anti-β-actin antibody produced in mouse (A2228, Sigma) was used. After washing with TPBS, the membranes were incubated with HRP conjugated secondary antibody (#711-035-152, Jackson ImmunoResearch Laboratories, West Grove, PA or #172-1011, BioRad, Hercules, CA). The labeled HRP was detected by ECL™ Western Blotting Detection Reagents (GE Healthcare UK Ltd, Buckinghamshire, England) and LAS3000 (Fujifilm, Tokyo, Japan). Band intensities were quantified using ImageJ software (NIH, Bethesda, MD).

Flow cytometry

Cells were washed with PBS(-) and harvested by trypsinization. After inactivation of trypsin by adding MEM containing FBS, cell suspensions (1×10^6 cells/ml) were prepared with PBS(-). After adding 1.2% TritonX-100-containing PBS(-) (final concentration 0.2%) and pipetting the sample, cells were passed through nylon mesh to remove debris. The cells were then treated with RNaseA solution (5 μg/ml) at 37°C for 30 min and stained with 6.7 μg/ml propidium iodide at 37°C for 20 min. After being passed through nylon mesh, samples were analyzed by BD FACSCalibur and BD CellQuest software (Becton Dickinson, Franklin Lakes, NJ).

Methyl methanesulfonate sensitivity

MMS sensitivity was examined by MTS assay or a relative cell growth assay. For the MTS assay, a cell suspension (100 μl) containing 20 000 cells was seeded into each well of a 96-well microplate. After incubation for 3 h, the culture medium was changed to MEM containing an appropriate amount of MMS (Sigma, M4016). After treatment for 1 h at 37°C, the culture medium was renewed to fresh MEM and cell survival was determined by colorimetric analysis with MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] using CellTiter 96® Aqueous One Solution (Promega, Madison, WI). The relative cell growth assay was performed as described by Sobol *et al.* [9]. Briefly, 35 000 cells were seeded onto 35-mm dishes and incubated for 16 h.

After washing with Hanks' Balanced Salt solution (Sigma), the cells were treated with MEM containing MMS (0 to 2 mM) for 1 h at 37° C. After MMS treatment, the cells were incubated in fresh MEM for 3 days. Relative growth was determined by cell counting.

RESULTS

Apex knockdown had little effect on the viability of PolBKOs immediately after MMS treatment

Wild-type MEFs (WT) and PolB-knockout MEFs (PolBKOs) were transfected with shRNA-expression vectors for Apex knockdown. All clones isolated from Apex-knockdown MEFs (ApexKD1, ApexKD2) and Apex-knockdown/PolB-knockout MEFs (AKDBKO1, AKDBKO2) expressed lower levels of Apex protein compared with WT cells (Fig. 1A). Interestingly, PolBKOs also expressed a lower level of Apex. Only ApexKD1 cells showed a significant fraction of a sub-G1 DNA population (Fig. 1B) [16]. While AKDBKO1 cells had much lower Apex expression (34% of WT MEFs), the cell population contained essentially no sub-G1 fraction.

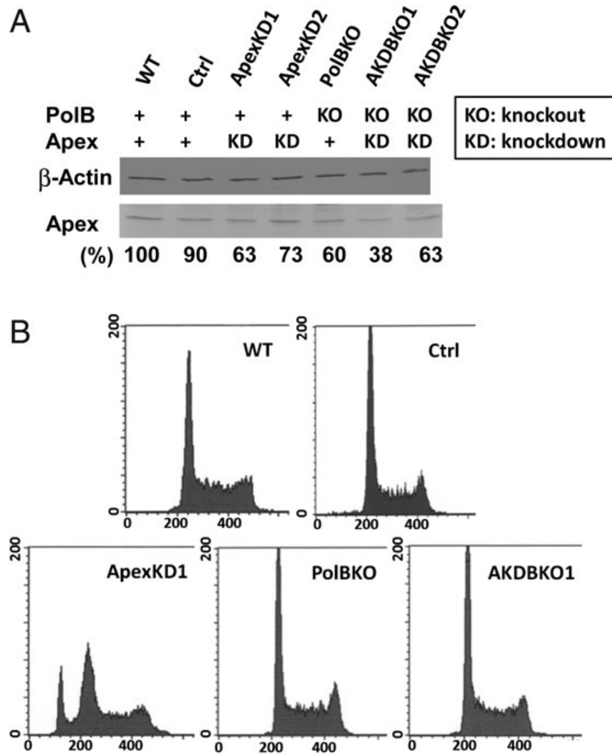

Fig. 1. Cellular Apex protein levels and cell-cycle distributions analyzed by flow cytometry of Apex-knockdown MEFs. A. Western blot analyses of Apex protein expression. Relative Apex levels in the knockdown clones are shown under the image. Each value is an average of two experiments. B. Cell cycle histograms of the knockdown clones. Histograms display PI fluorescence (DNA content) vs cell number. WT = Wild type, Ctrl = Empty vector-introduced, ApexKD1 and 2 = Apex-knockdown clones 1 and 2, PolBKO = PolB-knockout, AKDBKO1 and 2 = Apex-knockdown/PolB-knockout clones 1 and 2.

MMS sensitivity of the MEFs was examined by MTS assay immediately after MMS treatment. As illustrated in Fig. 2, control MEFs (Ctrl, empty vector) showed resistance to MMS, and PolBKOs showed significantly elevated sensitivity. WT cells showed a similar curve to Ctrl MEFs (data not shown). Apex knockdown had essentially no effect on cell viability in the presence or absence of PolB. Thus, Apex knockdown did not affect the number of viable cells immediately after MMS treatment.

Apex knockdown decreased the growing fraction of PolBKOs after MMS treatment

MMS sensitivity of MEFs was assayed 3 days after treatment using a relative cell growth assay. As reported previously, Ctrl cells were less sensitive at a low concentration of MMS (Fig. 3A). In contrast to the results of the MTS assay (Fig. 2), Apex knockdown had a significant effect on MMS sensitivity in the presence or absence of PolB. ApexKD and AKDBKO cells showed higher MMS sensitivity than Ctrl and PolBKOs, respectively (Fig. 3). At low MMS concentrations, curves for both ApexKD and AKDBKO cells showed an exponential relationship (Fig. 3). These results indicate that MMS sensitivity depends on Apex protein levels and suggest that resistance at low concentrations of MMS is due to an Apex-dependent/PolB-independent repair mechanism.

DISCUSSION

It is generally accepted that MMS-induced DNA damage is mainly repaired by Sn-BER. However, PolB-knockout MEFs showed resistance to low concentrations of MMS, and this resistance cannot be fully explained by the activities of PolL. In this study, we investigated the MMS sensitivity of Apex-knockdown/PolB-knockout MEFs, which should be deficient in both Sn-BER and Lp-BER, to examine the mechanism contributing to the resistance.

We prepared several Apex-knockdown MEFs using shRNA-expression vectors; however, the lowest Apex level achieved was 70% that of WT cells. It is possible that the non-tumor cells were unable to tolerate efficient and continuous Apex knockdown, unlike HeLa

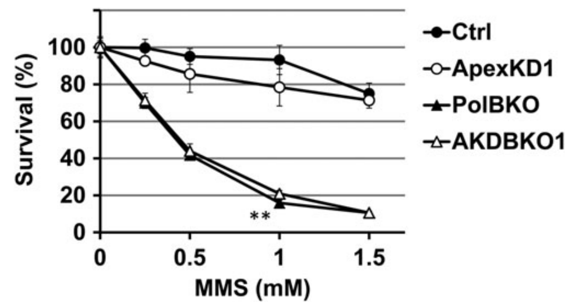

Fig. 2. A decrease in Apex had little effect on cell viability immediately after MMS treatment in the presence or absence of PolB. The cell viability of MEFs after MMS treatment was determined by MTS assay. Each point is the average of four experiments, and error bars represent the standard deviation from the mean. Error bars are shown when larger than symbols.

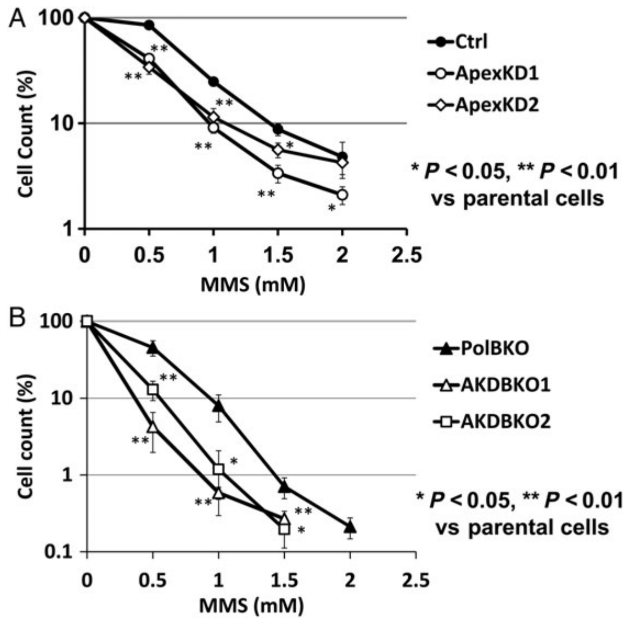

Fig. 3. A decrease in Apex reduced cell growth significantly in the presence or absence of PolB. Effects of Apex knockdown on cell growth after MMS treatment in the presence (A) or absence (B) of PolB. Each point is the average of four experiments. Error bars represent the standard deviation of the mean.

cells [7]. In fact, Izumi *et al.* demonstrated that lack of AP endonuclease elicited apoptosis in MEFs within 24 h [17]. Alternatively, in the selection process of shRNA-expression vector transfectants, more proliferative cells expressing higher Apex might have become dominant. Interestingly, PolBKOs expressed a lower level of Apex protein than control cells (Fig. 1A). The exact reason for this observation is unclear, but comparison of ApexKD and PolBKO cells emphasized the remarkable effect of PolB deficiency on cell survival. The MMS sensitivities were similar up to 1.0 mM, but PolBKOs showed significantly higher sensitivity at >1.5 mM MMS (Fig. 3), suggesting that PolL was unable to substitute for PolB deficiency for repair in response to >1.0 mM MMS. At low concentrations of MMS, the AKDBKO1 cells, which expressed lower Apex than ApexKD1 and PolBKO cells, showed much higher MMS sensitivity. These results suggest that resistance to low concentrations of MMS resulted from an Apex-dependent repair pathway.

Although the Lp-BER pathway is a possible candidate for Apex-dependent repair or tolerance, PolB plays a role not only in Sn-BER but also in Lp-BER pathways in mouse fibroblasts [18]. Therefore, we could not simply ascribe the enhancement of MMS-sensitivity to Lp-BER suppression. It has been reported that both deficiency and overexpression of methylpurine-DNA glycosylase (Mpg), which mainly removes methylated bases formed by MMS, induced chromosome aberrations after treatment with an alkylating agent [19–23], suggesting that levels of BER-related proteins are strictly controlled. In fact, the levels of Mpg, Apex, Fen1 and Pcna, which are required in the Lp-BER pathway, decreased in the PolBKOs used in this study (unpublished data), indicating that the decrease in Apex as well as the suppression of Mpg, Fen1 and Pcna proteins might contribute to the hypersensitivity of AKDBKO1. Although it is conceivable that the Apex-dependent/PolB-independent repair pathway may be involved in the resistance at low MMS concentrations, further studies are required to investigate the role of other known effects of Apex protein on the tolerance.

A sub-G1 cell population was observed in ApexKD1 cells (Fig. 1B). Because the appearance of sub-G1 cells resulting from the knockdown of AP endonuclease in human cells was recovered by expression of Apn1, yeast AP endonuclease lacking Ref1 activity [16], the sub-G1 cells in Fig. 1B were thought to appear together with a decrease in AP endonuclease activity. On the other hand, Izumi *et al.* concluded that both the DNA repair and acetylation-mediated gene regulatory function of APE1 are essential for mouse cell viability [17]. It is possible that other functions of Apex besides AP endonuclease activity might be related to the appearance of sub-G1 cells in mice. However, because the sub-G1 cells were absent in ApexKD2, in which the Apex level was reduced to about the same as ApexKD1, and in AKDBKO1 cells expressing greatly reduced levels of Apex (Fig. 1B), it is possible that the sub-G1 population stemmed from causes other than Apex knockdown. Alternatively, since the sub-G1cells disappear in AKDBKO1 and PolBKO in which a lower level of Apex protein was observed, it is possible the lack of PolB might correct the imbalance in the BER system.

In conclusion, in the present study, the results suggest that the Apex-dependent/PolB-independent pathway exists to repair MMS-induced damage.

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