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Defining the DNA mismatch repair-dependent apoptotic pathway in primary cells: Evidence for p53-independence and involvement of centrosomal caspase 2

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ABSTRACT

Many studies have shown that DNA mismatch repair (MMR) has a role beyond that of repair in response to several types of DNA damage, including ultraviolet radiation (UV). We have demonstrated previously that the MMR-dependent component of UVB-induced apoptosis is integral to the suppression of UVB-induced tumorigenesis. Here we demonstrate that Msh6-dependent UVB-induced apoptotic pathway is both activated via the mitochondria and p53-independent. In addition, we have shown for the first time that caspase 2, an initiator caspase, localizes to the centrosomes in mitotic primary mouse embryonic fibroblasts, irrespective of MMR status and UVB treatment.

1. Introduction

DNA mismatch repair (MMR) plays a critical role in the maintenance of genomic stability through the repair of mis-paired bases and insertion/deletion loops (IDLs) that occur during DNA replication [1]. The first step in MMR is the binding to the site of DNA error: the MSH2/MSH6 heterodimer binds mismatches and small IDLs, while the MSH2/MSH3 heterodimer binds larger IDLs. The importance of MMR to tumor avoidance is illustrated by Lynch syndrome (formerly termed HNPCC); loss of a key MMR protein (e.g., MSH2, MLH1 and MSH6) culminates in a distinct tumor spectrum including colon and endometrial tumors. Notably, colon tumors are the predominate tumor associated with loss of either MSH2 or MLH1, while endometrial tumors are associated with a loss of MSH6.

In addition to its role in post-replicative DNA repair, MMR proteins are integral to the cellular response to exogenous DNA damage; responses include processing of DNA lesion, as well as apoptosis and cell cycle arrest [2]. Lesions can be divided into three groups: (i) lesions both bound and repaired by MMR; (ii) lesions that are bound, but not repaired by MMR; and (iii) lesions that are not bound by MMR. DNA damage induced by SN1-type alkylating agents (e.g., MNNG) is a classic example of MMR-processed lesions. MMR-deficiency confers resistance to MNNG, as manifested by decreased induction of apoptosis, decreased induction of G_2M checkpoint arrest, and increased DNA mutation accumulation [3–7].

In contrast, MMR binds to UVB-induced mismatch/photoproduct compound lesions [8,9] (caused by translesion synthesis across the original lesion), but does not participate in the removal of this adduct (reviewed in [10]). In recent years several studies by our laboratory and those of others have demonstrated a role for MMR in the cellular response to UV-induced DNA damage [11–17]. Using both primary cultures of MEFs and *in vivo* keratinocytes, we have demonstrated that the loss of either Msh2 or Msh6 results in a 2-fold reduction in UVB-induced apoptosis [11–13]; also, the reduced numbers of in apoptotic keratinocytes *in vivo* (sunburn cells) is associated with increased levels of residual photoproducts (thymine-dimers) [13] and accelerated presentation and increased severity of squamous cell carcinoma tumorigenesis [13–15]. Other research groups have demonstrated clearly that Msh2-dependent apoptosis can be uncoupled from Msh2-dependent repair, and that mice deficient only in Msh2-dependent apoptosis remain predisposed to tumorigenesis, albeit at a reduced rate than *Msh2*-null mice (deficient in both functions)

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[18,19]. Together, these data indicate that key MMR proteins play a role in the cellular responses to UVB-induced DNA damage, specifically in the promotion of apoptosis, and the avoidance of UVB-induced carcinogenesis.

Despite the clear association between MMR and the promotion of apoptosis in response to some forms of DNA damage, the mechanism by which MMR proteins affect the induction of apoptosis is not known. In order to characterize further the MMR-dependent component of UVB-induced apoptosis, we sought to delineate the affected apoptotic pathway. We found that MMR-dependent apoptosis occurred *via* the intrinsic/mitochondrial pathway, but that it was independent of p53. Moreover, our data demonstrated that both the Msh2/Msh6 heterodimer and caspase 2 were present in the centrosome. These data introduce the novel idea that MMR/caspase 2 may promote an apoptotic pathway that emanates from the centrosome this nuclear structure.

2. Materials and methods

2.1. Cell culture

Primary MEFs were generated and cyropreserved as described previously [11–13]. Once retrieved from liquid nitrogen storage, cells were expanded for 2–3 days before plating for UVB experiments (not exceeding 1:4 split), and were not grown beyond 7 days post-plating.

2.2. UVB irradiation

Primary MEFs previously plated on tissue culture plates were washed twice with PBS, and the PBS removed. Cells were exposed to UVB (290–320 nm) from a bank of six unfiltered UVB bulbs (FS20T12/UVB-BP, Light Sources Inc., Orange CT); the tissue culture lid was kept on the plates to maintain sterility and to filter out UVB. The intensity of the UVB source was measured using IL1700 radiometer with a SED 240/UVB-1/W detector (International Light, Newburyport, MA), taking into account the passage of the light through the tissue culture lid. Following UVB irradiation, warmed DMEM plus 20% FBS was replaced immediately on the MEFs.

2.3. Apoptosis assay of primary MEFs

Msh6$^{+/+}$ and *Msh6*$^{-/-}$ primary MEFs were generated, cultured and UVB-irradiated for experiments as described above. Adherent and suspension MEFs were collected 24 or 48 h following UVB irradiation. Cells were stained using an Annexin-FITC Apoptosis Kit (BD Pharmingen, San Diego, CA). The percentage of apoptotic cells was determined by flow cytometry using Becton Dickenson FACScalibur and BD CellQuest™ program (Becton Dickenson, San Jose, CA).

2.4. Immunoblot analysis

Msh6$^{+/+}$ and *Msh6*$^{-/-}$ primary MEFs were harvested and cell pellets were resuspended in loading buffer (100 mM Tris pH 6.8, 16% glycerol, 3.2% SDS) and sonicated. Lysates were separated by discontinuous SDS polyacrylamide gel electrophoresis and electrotransferred onto 0.45 μM Immobilon-P PVDF membrane (Millipore, Etobicoke, ON Canada) in Tris/glycine SDS transfer buffer. Blots were blocked in 5% (wt/vol) dry milk/TBST. Primary antibodies used were as follows: Caspase 2 (#MAB3501, Chemicon, Temecula, CA), Caspase 2 (#611023, BD Transduction, Mississauga, ON, Canada), Caspase 2 (#3027-100, Biovision, Mountain View, CA), BAX (#556467, BD Pharmingen, San Diego, CA), Cytochrome c (#S2050, BD Biosciences/Clontech, Mountain View, CA), Caspase 9 (#9504, Cell Signaling Technology, Beverly, MA). Antibody binding was visualized using the appropriate horseradish-peroxidase conjugated secondary antibody and ECL (Amersham Bioscience, Picataway, NJ) on Fuji SuperRX film (Fujifilm, Stamford, CT). The Quantity One densitometry program (Biorad Laboratories, Hercules, CA) was utilized to account for loading variations. The densitometry values for the proteins of interest were normalized either to γ-tubulin or to total protein levels as determined by amido black stain. Immunoblots were replicated 3 times; a representative experiment is shown.

2.5. Mitochondrial membrane sensor assay

Harvested MEFs were stained using the ApoAlert™ Mitochondrial Membrane Sensor Kit (Clontech Laboratories, Inc., Mountain View, CA) according to manufacturer instructions and analyzed by flow cytometry using Becton Dickenson FACScalibur and BD CellQuest™ program (Becton Dickenson, San Jose, CA). Mean levels of mitochondrial membrane depolarization were compared using the student's t-test.

2.6. Cytochrome c release assay

Untreated and UVB-treated *Msh6*$^{+/+}$ and *Msh6*$^{-/-}$ primary MEFs and medium were harvested by cell scraping. Mitochondrial and cytosolic fractions were obtained by using the ApoAlert™ Cell Fractionation Kit (Clontech Laboratories, Inc., Mountain View, CA) according to manufacturer instructions.

2.7. Cellular fractionation

Untreated and UVB-treated *Msh6*$^{+/+}$ and *Msh6*$^{-/-}$ primary MEFs and medium were harvested by cell scraping. Cytosolic, nuclear, membrane/particulate, and cytoskeletal fractions were obtained by using the FractionPREP™ Cell Fractionation System (Biovision Research Products, Mountain View, CA) according to manufacturer instructions.

2.8. Caspase inhibitor treatment of primary MEFs

Msh6$^{+/+}$ and *Msh6*$^{-/-}$ primary MEFs were generated and cultured as above. Medium was removed and cells were rinsed with PBS. Caspase inhibitor against caspase 2 (Z-VDVAD-FMK) or caspase 9 (Z-LEHD-FMK) was added to fresh medium at a concentration of 100 μM and incubated at 37 °C for 2 h. Medium was removed and cells were rinsed with PBS and then UVB-irradiated as described above. Medium with caspase inhibitor was replaced and cells were incubated at 37 °C for 24 h and then adherent and suspension MEFs were collected. As the caspase inhibitors were solubilized in DMSO, the non-UVB-treated control MEFs were cultured with the corresponding volume of DMSO.

2.9. Centrosome isolation from primary MEFs

Untreated *Msh6*$^{+/+}$ and *Msh6*$^{-/-}$ primary MEFs were generated and cultured as above. MEFs were incubated with DMEM containing 1 μg of cytochalasine D/ml and 0.2 μM nocodazole for 1 h at 37 °C to depolymerize the actin and microtubule filaments. MEFs were harvested by trypsinization and centrosomes were isolated as described in [20].

2.10. Immunofluorescence

Msh6$^{+/+}$and *Msh6*$^{-/-}$ primary MEFs were split onto coverslips coated with poly-L-lysine (0.1 mg/mL; Sigma, Saint Louis, MO) and were fixed with methanol at various timepoints after treatment. Cells on coverslips were blocked in 1% BSA (Sigma, Saint