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Enhanced mtDNA repair and cellular survival following oxidative stress by targeting the hOGG repair enzyme to mitochondria.

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Running title: Enhanced survival after oxidative stress by increased mtOGG.

SUMMARY

Oxidative damage to mtDNA has been implicated as a causative factor in many disease processes and in aging. We have recently discovered that different cell types vary in their capacity to repair this damage, and this variability correlates with their ability to withstand oxidative stress. To explore strategies to enhance repair of oxidative lesions in mtDNA, we have constructed a vector containing a mitochondrial transport sequence upstream of the sequence for human 8-oxoguanine glycosylase. This enzyme is the glycosylase/AP lyase that participates in repair of purine lesions, such as 8-oxoguanine. Western blot analysis confirmed this recombinant protein was targeted to mitochondria. Enzyme activity assays showed that mitochondrial extracts from cells transfected with the construct had increased enzyme activity compared to cells transfected with vector only, while nuclear enzyme activity was not changed. Repair assays showed that there was enhanced repair of oxidative lesions in mtDNA. Additional studies revealed that this augmented repair led to enhanced cellular viability as determined by reduction of tetrazolium compound to formazan, Trypan blue dye exclusion, and clonogenic assays. Therefore, targeting of DNA repair enzymes to mitochondria may be a viable approach for the protection of cells against some of the deleterious effects of oxidative stress.

INTRODUCTION

A variety of diseases have been associated with alterations in mitochondrial DNA (mtDNA) including diabetes mellitus (1,2), Alzheimer's disease (3-6), and Parkinson's disease (7-11). Additionally, deleterious phenotypes associated with the normal process of aging have been correlated with these lesions (12-17). Because errors in mtDNA lead to erroneous translation of important subunits of the electron transport chain, the result can be a deficiency in the production of ATP along with the "leak" of electrons from the various protein complexes involved in oxidative phosphorylation. These electrons cause damage to proteins, lipids, and DNA through formation of intermediate reactive oxygen species (ROS). As more damage is sustained, the mitochondria become more dysfunctional, and a self-propagating detrimental cycle ultimately ensues. Finally, if and when sufficient damage is produced, an apoptotic program can be initiated in the affected cell or the cell may die by necrosis (13, 18, 19).

Mitochondrial DNA is particularly susceptible to damage by ROS because of its close proximity to the electron transport chain and its lack of protective histones. Previous studies by our laboratory and others (20-24) show that this DNA is considerably more vulnerable to exogenously-generated damage than is nuclear DNA. Oxidative damage to mtDNA can be repaired efficiently by some cell types (24-26). However, other cell types such as certain types of glial cells and neurons (24,27) are much less proficient at repairing this damage. To date, the mechanisms involved in this repair have not been well defined. Since the discovery of mammalian 8-oxoguanine glycosylase (OGG) (28), variant splices of OGG, MYH (the human homolog of *E.coli* MutY, which excises mispaired adenine opposite 8-oxoguanine), and NTH1 (the human homologue of *E.coli* endonuclease III) have been localized to mitochondria (29). Recent evidence shows that there is an increase in the level of 8-oxoguanine lesions in mtDNA

with age (16). Another study shows that there is an age-associated increase specifically in OGG activity in mitochondria and not in other repair enzymes (30). Together, this suggests that the DNA repair system in mitochondria may be differentially regulated and that OGG enzyme may play a pivotal role in this regulation. Still, there is much to be learned about the components and processes involved in mtDNA repair of oxidative damage.

A plausible explanation for why various cell types possess different inherent capacities for repairing mtDNA (24-27) is that there are differences in the expression of specific components involved in DNA repair. To explore this possibility, we sought to increase the DNA repair capacity of a cell line that we have found to be relatively inefficient at repairing oxidative damage to its mtDNA. Because 8-oxoguanine has been considered to be one of the most mutagenic of oxidative lesions in DNA due to its strong tendency to mispair with adenine (31), we targeted the glycosylase/AP-lyase that repairs this lesion in the nucleus of human cells, hOGG, to the mitochondria. We investigated the effect of the targeted recombinant protein on mtDNA repair, cell survival, and the ability to proliferate. The results indicate that this protein enhances the repair of oxidative damage to mtDNA and increases the capacity for cells to survive and continue to divide following an oxidative insult.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections: HeLa cells were obtained from ATCC. The cells were maintained in Eagle's minimal essential medium with Earle's salts (Gibco BRL), supplemented with 10% fetal bovine serum (HyClone Laboratories), 50 µg/mL penicillin/streptomycin (Sigma), and 2 mM L-glutamine (Gibco BRL) in 5% CO₂ at 37°C, and passaged every 3-4 days. For transfections, cells were grown in 75 cm² flasks until they reached 75% confluence. They were then transfected with Fugene 6 reagent according to manufacturer's recommendations.

After 24 h, selection with 0.6 mg/mL G418 (geneticin) ensued. After 2 weeks of selection, the cells were maintained in 0.4 mg/mL G418.

OGG construct: Oligonucleotides were designed to serve as primers to amplify OGG1 from a cDNA plasmid. Additionally, the 5' primer, GGAATTCATGTTGAGCCGGGCAGTGTGCGGCACCAGCAGGCAGCTGGCTCCGGCTTTGGGGTATCTGGGCTCCAGGCAGATGCCTGAATTACCCGAAGTT, contained the mitochondrial targeting sequence (MTS from MnSOD, 32) and an EcoRI restriction site, and the 3' primer, CGCCGCTCGAGGCCTTCCGGCCCTTTGGA, contained an XhoI restriction site. The cDNA was amplified using a high-fidelity thermostable DNA polymerase by PCR in a thermal cycler under the following conditions: 30 sec denaturation (94°), 1 min annealing (55°), 2 min extension (72°). The resulting PCR fragment consisted of an EcoRI site, the MTS, the OGG coding region, and an XhoI site. The PCR product was subjected to double restriction enzyme digest with EcoRI and XhoI overnight at 37°C. The restriction fragment was sub-cloned into the EcoRI and XhoI sites of pcDNA3.0neo and sequenced to confirm fidelity. The predicted protein is approximately 39 kD.

Preparation of Cellular Fractions: Three 75 cm² flasks of each cell type (MTS-OGG- and control vector-transfected) at confluence were harvested and treated with ice-cold digitonin (325 mM digitonin, 2.5 mM EDTA, 250 mM mannitol, 17 mM MOPS, pH 7.4) for 80 sec. The lysed cells were then added to mannitol-sucrose buffer for a final strength of 1X (210 mM mannitol, 70 mM sucrose, 5 mM EDTA, 5 mM tris, pH 7.5). The ice-cold suspension was then centrifuged for 10 min at 800 x g to pellet nuclei. The supernatant was saved, the pelleted material was resuspended in 1X mannitol-sucrose, and centrifugation repeated. This was repeated 3 more times. The combined supernatants were then centrifuged to pellet any

remaining nuclei, and the resulting supernatant was centrifuged at 10,000 x g to pellet mitochondria. The combined supernatants were concentrated (Amicon protein concentrators) for cytosolic fractions. Isolated mitochondria and nuclei were suspended in a buffer of 20 mM HEPES, pH 7.6, 1 mM EDTA, 5 mM dithiothreitol, 300 mM KCl, and 5 % glycerol. These preparations were briefly sonicated on ice, and 5 μ L of protease inhibitor cocktail from Sigma (for mammalian cell extracts, 100 mM AEBSF, 4 mM bestatin, 1.4 mM E 64, 2.2 mM leupeptin, 1.5 mM pepstatin, and 80 μ M aprotinin) was added per mL buffer. The fractions were centrifuged once more at 5,000 x g to pellet any remaining cell debris, and supernatant protein was used for Western blots and OGG activity assays. Protein concentrations were determined using the Bio-Rad protein dye micro-assay according to manufacturer's recommendations (Bradford method).

Western Blots: The organelle-enriched fractions from each cell type were lysed and quantitated as described above. Fifty μ g of each sample was loaded onto 12% SDS-polyacrylamide gels and electrophoresed to resolve proteins. The proteins were then transferred to Immobilon P PVDF transfer membranes (Millipore) and blocked in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 6% nonfat dry milk. Incubation with 1:1000 dilution of anti-hOGG primary antibody, which was kindly provided by Dr. S. Mitra, was overnight at 4°C in the same solution. The membrane was then washed in 50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20 4 times and then in the same solution without Tween-20 2 times. The horseradish peroxidase-labeled anti-rabbit secondary antibody was incubated with the membrane for 4 hr at 4°C, the washes were repeated as before, and the membrane was reacted with chemiluminescent reagents (SuperSignal, Pierce) and processed for autoradiography. Monoclonal cytochrome C antibody was purchased from Pharmingen, and blotting procedures were as described above with anti-

mouse secondary antibody.

OGG Activity Assays: A 24-mer oligonucleotide with 8-oxoguanine at the 10th position (Trevigen) was end-labeled. An identical 24-mer without 8-oxoguanine also was used in parallel reactions. The labeling reaction contained: 5 pmol single strand 8-oxoguanine oligonucleotide, 5 pmol γ -³²P, T4 polynucleotide kinase, and appropriate kinase buffer in a total volume of 20 μ L, 37°C for 30 min, 90°C for 2 min. Complementary oligonucleotide (also 5 pmol) was then added to form duplex DNA. Equal amounts of protein from the fractions isolated from both cell types were then used in assays with labeled duplex oligonucleotide. Activity assays contained: 0.2 pmol labeled duplex oligonucleotide, 3 μ L 10X REC buffer (100 mM HEPES, pH 7.4, 1 M KCl, 100 mM EDTA, 1 mg/mL BSA), and organelle extracts or control formamidopyrimidine DNA glycosylase (Fpg) enzyme in a total volume of 30 μ L, 37°C for 1 hr. Organelle extracts contributed less than 20% of the total reaction volume (50 μ g mitochondrial, 20 μ g nuclear, 50 μ g cytosolic protein). Bromophenol blue dye was then added, and reaction contents were resolved on 20% acrylamide, 8M urea gels in 1X TBE.

Drug Exposure: Menadione (Sigma), a redox cyler (33,34), was dissolved in Eagle's minimal essential medium with Earle's salts only (no serum), at a concentration of 400 μ M for DNA repair studies. This was applied to MTS-OGG-transfected and control-transfected cells at approximately 75% confluence in 60 mm culture plates for 1 hr in 5% CO₂ at 37°C. Cells were then either lysed immediately (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% SDS, and 0.3 mg/mL proteinase K) or regular growth media was replaced and repair time allowed in the incubator before lysis of cells. Control cultures were exposed to drug diluent only. Lower doses of menadione were used in clonogenic assays due to higher sensitivity of sparsely plated cells.

Southern Blots: Cell lysates from the treatment described above were incubated

overnight at 37°C, 0.2 volume of 5M NaCl was added, and high molecular weight DNA was extracted with equal volumes of chloroform 3 times, followed by precipitation with ammonium acetate and ethanol. DNA was then resuspended in dH₂O, treated with RNase (final concentration 1 mg/mL) for 2 hr, and digested with XhoI overnight at 37°C. Digested samples were precipitated, resuspended in TE buffer, and precisely quantified using a Hoefer TKO 100 minifluorometer and TKO standard kit. Samples containing 5 µg of total DNA were heated for 15 min at 70°C and cooled at room temperature. NaOH then was added to a final concentration of 0.1 N, and samples were incubated at 37°C for 15 min. Samples were then mixed with alkaline loading dye and loaded onto a horizontal 0.6% alkaline agarose gel and electrophoresed at 30V (1.5V/cm gel length) for 16 hr. After ethidium bromide staining to confirm even loading and standard gel washes, the DNA was transferred to Zeta-Probe GT nylon membranes (Bio-Rad). The membranes were cross-linked and hybridized with ³²P-labeled human mtDNA specific PCR-generated probe. Hybridization and subsequent washes were performed according to manufacturer's recommendations. DNA damage and repair were determined as previously described (25, 35). The neutral Southern Blot was performed the same way except that there was no alkaline pretreatment of samples and no NaOH in the loading dye, the 0.6% agarose gel, or the electrophoresis buffer. DNA samples were digested with XhoI and EcoRI, and hybridization was performed with ³²P nick-translated MTS-OGG fragment.

Viability studies: The CellTiter 96 assay (Promega), which assesses mitochondrial function, was done according to manufacturer's recommendations 24 h after 1 h exposure to 200, 300, 400, and 500 µM of menadione. Briefly, the reagent is added to culture wells, and the cells are incubated for 2 hours. The tetrazolium compound is converted to a colored formazan product that is measured at 490 nm in a 96-well plate reader. Trypan blue exclusion studies were also

performed on controls and samples treated with menadione and allowed 6 h recovery in growth media. Additionally, a clonogenic survival assay also was employed to determine survival as well as the capacity of surviving cells to proliferate. MTS-OGG transfectants and control vector (pcDNA3) transfectants were carefully counted with a hemocytometer and 400 cells were plated into each 60 mm culture plate. These cells were allowed 24 hr in normal culture medium to adhere, and then they were exposed to menadione exactly as described above except that concentrations of 25, 50, 75, and 100 μ M were utilized due to the increased sensitivity of cells to oxidative stress when plated at the low density required for the clonogenic assay. Each cell type was assayed at control (no menadione) and all 4 concentration levels in triplicate. After exposure for 1 hr, normal culture medium was replaced and plates were incubated (5% CO₂ at 37°C) for 10 days. Then plates were rinsed with warm phosphate-buffered saline and fixed with a solution of 3 parts methanol: 1 part acetic acid for 10 min. Finally, the plates were stained with hematoxylin and colonies were counted.

Data analysis: All statistical analyses were performed using Student's T-test to compare individual means with significant differences at a confidence level of $p < 0.05$.

RESULTS

MTS-OGG transfection – In order to increase repair of oxidative damage in mtDNA, a construct with the human 8-oxoguanine glycosylase (hOGG) gene fused to the mitochondrial targeting sequence from human MnSOD (32) was prepared (Figure 1a). After transfection of HeLa cells with the MTS-OGG construct or control vector (pcDNA3) and 2 weeks of selection, DNA was isolated from pcDNA3- (vector only) transfected cells and MTS-OGG cells, and a neutral Southern blot was performed to check for integration of the transfected DNA. Figure 1b shows that the MTS-OGG sequence was recognized only in the MTS-OGG-transfected cells,

Figure 1
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where the predicted 1.1 kb band can be seen.

Detection of additional OGG protein in mitochondria – Mitochondrial, nuclear, and cytoplasmic fractions were isolated from vector only transfectants and MTS-OGG transfectants by differential centrifugation, and Western blots were performed with a polyclonal antibody to human OGG1. Figure 2 shows additional 39 kDa recombinant protein in the mitochondria of the MTS-OGG transfected sample. No differences in protein bands were detected in lanes containing nucleus- or cytosolic-enriched fractions. Even loading was confirmed with Coomassie staining. To further establish that the recombinant protein is in mitochondria, we also performed Western blot analysis for the mitochondrial protein cytochrome C and found it in the mitochondria (but not nucleus or cytosol) with equal amounts in both the vector and MTS-OGG samples (Figure 2). Thus, it can be concluded that the construct is functional in targeting additional human OGG to mitochondria.

Figure 2
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OGG Activity - In order to analyze the enzymatic activity of the additional OGG protein in the mitochondria of stable transfectants, an oligonucleotide cleavage assay was used. A 24-bp oligonucleotide with 8-oxoguanine at the 10th nucleotide was incubated with purified bacterial FPG (control) or extracts from isolated mitochondria or nuclei from MTS-OGG and vector-only transfectants, as described in the experimental procedures. Figure 3 shows the intact DNA and cleavage products from each of these reactions. Equal amounts of protein were used in each comparison between vector and MTS-OGG transfected cells. The mitochondrial extracts from MTS-OGG-transfected cells are better able to cleave the DNA than the control cells. The nuclear extracts, on the other hand, show equal enzyme activity levels. None of the extracts or purified FPG were able to cleave an identical oligonucleotide duplex with normal guanine at position 10. Based on this assay, we conclude that the additional OGG protein targeted to

Figure 3
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mitochondria is indeed functional in removal of 8-oxoguanine and strand cleavage.

DNA Repair Analysis – Because the MTS-OGG cells have an elevated level of mitochondrial human OGG, which contains lyase activity, it is possible that repair of damage to the sugar-phosphate backbone is enhanced. To test for this, dose response studies were performed using different concentrations of menadione, which redox cycles with complex I of the electron transport chain to form superoxide radical (33, 34). A concentration of 400 μM menadione produced an appropriate amount of lesions (~ 1 lesion per 10^4 normal nucleotides) in mtDNA from both MTS-OGG transfectants and control pcDNA3 transfectants. Damage to nuclear DNA was undetectable at this dose using Southern blots or quantitative extended length PCR (not shown). Therefore, repair experiments were performed in which MTS-OGG transfectants and pcDNA3 transfectants were exposed to 400 μM menadione, followed by either immediate lysis or lysis after repair intervals up to 6 h in normal culture medium. Control cultures were exposed to drug diluent only. DNA was isolated from the lysed cells, and quantitative Southern blots were performed to check overall damage levels and the subsequent repair of this damage. As shown in Figure 4a, the pcDNA3 transfectants did not repair an appreciable amount of the damage to their mtDNA within the initial 6 h following drug removal, whereas the MTS-OGG transfectants repaired most of the damage in this time interval. The average amount of repair for each cell type is shown in Figure 4b. Based on these results, it can be concluded that the additional OGG targeted to the mitochondria is a functional enzyme, and that mtDNA repair in these cells is more efficient than in the control transfectants.

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Viability Analysis – To investigate whether the observed increase in mtDNA repair translates into enhanced viability after oxidative insult, three assays were employed to evaluate the MTS-OGG transfectants as compared to the pcDNA3 transfectants. First, the mitochondrial

function of these cells was analyzed 24 h after exposure to the menadione diluent (serum free culture medium) or 200, 300, 400, and 500 μM menadione. Figure 5a graphically demonstrates the average of three independent experiments. A progressive decrease in the ability of mitochondria to reduce tetrazolium compound to formazan was seen with increasing dose of menadione. A significant difference in conversion to formazan was observed between MTS-OGG cells and those transfected only with the vector at the 500 μM dose. A Trypan blue exclusion assay was performed on cells treated likewise followed by 6 h of recovery time. A significantly greater percentage of the MTS-OGG cells were able to exclude the dye after 6 h (Figure 5b). However, because Trypan blue dye exclusion and tetrazolium reduction are only transient measures of viability and not necessarily indicative of long-term cell survival, a clonogenic survival assay also was utilized. Cells from the two transfected cell lines were carefully counted and 400 cells plated into 60 mm dishes. Due to the sparse plating conditions, these cells were more sensitive to the menadione than confluent cells. Therefore, the doses utilized in this assay were lower than the doses used for DNA repair studies. After 24 h in culture medium, the plates were treated with various doses of menadione for 1 h and then cultured for 10 days in normal culture medium. The resulting colonies represent cells that were not only viable, but also able to proliferate. Figure 6 reveals that MTS-OGG cells were significantly better able to produce colonies at all concentrations tested. These viability data establish that MTS-OGG transfectants are better able to survive an oxidative challenge than the control cells.

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DISCUSSION

To our knowledge, this is the first report to describe the targeting of a recombinant repair enzyme to mitochondria in an effort to correct deficient repair of oxidative damage in the DNA

in this organelle. The isolate of HeLa cells used for these studies was selected because it was discovered that these cells do not repair oxidative damage to their mtDNA proficiently, and that they grow well in culture so stable transfectants could be easily obtained. The Western blot data from isolated mitochondria confirm that the targeted DNA repair enzyme OGG actually localized to mitochondria. The MTS-OGG-transfected cells contained a 39 kDa molecular weight protein, which is the predicted size of the protein produced by the MTS-OGG construct. There was not a corresponding protein band in the vector-transfected cells. Further evidence that the recombinant protein actually is present in mitochondria is supplied by the finding that the mitochondrial protein cytochrome C is also present in the mitochondrial protein preparations. Differences in protein bands were not seen in nuclear or cytoplasmic extracts for MTS-OGG- or vector-transfected cells.

Because the transfected protein contains both mitochondrial and nuclear localization sequences, the compartmentalization of the recombinant protein is in question. We feel that the answer to this issue lies partially with the positioning of the targeting sequence. It has been reported that when multiple targeting sequences are present on a protein, the one closest to the N-terminus usually dominates. This was demonstrated for another repair protein, uracil glycosylase. When the MTS is at the N-terminal region, this protein localizes exclusively to mitochondria. However, when the MTS is deleted, the protein localizes to the nucleus (36). In our recombinant protein, the MTS is closest to the N-terminus. The targeting of proteins to mitochondria also seems to be dependent upon the strength of the MTS. OGG1-1a, which has been found to have a weak MTS, predominantly localizes to the nucleus. When the nuclear localization sequence is deleted, the protein goes to mitochondria (37). The MTS that we selected is from MnSOD, and has previously been found to be a strong MTS that effectively

directs other proteins to mitochondria (38). Thus, we believe that it is the combined effect of a strong MTS and a position near the N-terminus of the recombinant protein, which has selectively targeted this repair enzyme to mitochondria.

The enzyme activity and DNA repair studies show that the targeted repair protein is functional. Mitochondrial extracts from MTS-OGG-transfected cells had markedly increased enzyme activity than did parallel extracts from vector-transfected cells, when normalized for protein content, as determined by the use of an assay employing a duplex oligonucleotide substrate containing 8-oxoguanine. Moreover, the MTS-OGG-transfected cells were significantly more proficient at repairing oxidative damage in their mtDNA. In contrast, the enzyme activity for cleaving the 8-oxoguanine substrate was the same in nuclear extracts from both MTS-OGG- and vector-transfected cells. Therefore, it can be concluded that the recombinant enzyme was selectively targeted to mitochondria in an active form and that it has a profound effect upon the repair of oxidative damage in the DNA in this organelle. Additionally, when combined with viability studies, it is readily apparent that increased repair of oxidative lesions in mtDNA renders cells more resistant to the lethal effects resulting from heightened oxidative stress.

The difference in cellular viability following menadione-induced damage is surprising if hOGG1 acts only on 8-oxoguanine. However, there have been reports of independent AP lyase activity associated with the human and murine forms of this enzyme (39,40). A very recent study on the murine homologue, mOGG1, concludes that it is a “bifunctional DNA glycosylase with uncoupled AP lyase activity.” A variety of lesions are shown to be substrates (40). The authors also suggest possible “direction of assembly of an AP site complex” including AP endonuclease and DNA polymerase by the OGG enzyme. While the independent lyase activity is lower than the glycosylase activity, overexpressed quantities of the enzyme may affect the

processing of AP sites in DNA. We believe that this independent lyase activity is responsible for the repair data seen in Figure 4 in which the removal of alkali sensitive sites is enhanced in the MTS-OGG cells. Because oxidative stress causes a variety of damages to DNA, many which are not substrates for OGG1, it can be questioned why increased expression of OGG1 is protective. We feel that the answer is that a variety of damages contribute to the demise of the cell. If one group of lesions is diminished, the cell does not cross the threshold into irreversible cell death. The present work does not rule out the possibility that other enzymes also may be protective or that combinations of enzymes may enhance viability even more. Indeed, these are currently active areas of investigation in our laboratory.

We, and others subsequently, have identified a variety of cells that efficiently repair oxidative damage to mtDNA generated by several agents, including menadione (20,25,35). Therefore, the ability to proficiently repair oxidative damage does not appear to be dependent upon the type of damaging agent used. As can be seen from the present results, this repair is markedly enhanced by the overexpression of a DNA glycosylase/AP lyase. We have converted a cell type with a mtDNA repair deficient phenotype to a proficient one, and the result is increased survival after menadione treatment.

Although the identification of the exact mechanisms whereby an alteration in mtDNA repair affects cell viability remain to be fully elucidated, we believe that enhanced repair works to restore a normal lesion equilibrium in mtDNA. When oxidative stress increases, there is a rise in the oxidative lesions in mtDNA. At some point as this stress progresses, there will be more lesions in the mtDNA than the endogenous repair system can remove in order to keep the number of lesions in the mitochondrial genome at a manageable level. This will cause an alteration in the transcription of this genome, either through base mispairing to cause defective

transcripts or decreased transcription resulting from polymerase blocking. Either process will alter the flow of electrons by changing key electron transport complexes such that more ROS will be produced (41). This increased production of ROS will exacerbate the ongoing oxidative stress in the cell and lead to altered cellular functions due to elevated oxidative damage and decreased ATP production. As this process continues, increased cellular death will transpire through either apoptotic or necrotic mechanisms. By enhancing the ability of the cell to repair the increase in lesions in its mtDNA resulting from heightened oxidative stress, the lesion equilibrium in mtDNA can be maintained within normal limits and the vicious cycle initiated by elevated oxidative stress can be prevented. Our recent studies with glial cells have shown that the repair of oxidative damage in mtDNA correlates well with the ability of different glial cell populations to resist the induction of apoptosis (42). In a similar manner, we feel that the increase in mtDNA repair capacity in the present study works to keep the lesion equilibrium within a normal range following the oxidative stress induced by menadione. Because increased lesions in mtDNA have been associated with the pathogenesis of many chronic diseases, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and noninsulin-dependent diabetes mellitus (1-11), we feel that targeting of repair enzymes to mitochondria may be a feasible gene-therapy strategy for either preventing or delaying the onset of these diseases. Additionally, this approach may prove useful for protecting normal cells during certain forms of cancer chemotherapy, and for preventing deleterious phenotypes associated with the normal process of aging. The present work represents the initial steps in the development of this protective strategy.

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FIGURE LEGENDS

Fig. 1. **MTS-OGG DNA is transfected into HeLa cells.** A. The construct was prepared with a mitochondrial targeting sequence from human MnSOD upstream (5') from the human OGG gene in the pcDNA3 vector. B. The OGG sequence is detectable in transfected cells only. A Southern blot was performed using total DNA isolated from untransfected cells, cells transfected with the MTS-OGG construct, or the vector alone. The probe corresponds to the MTS-OGG segment of the construct.

Fig. 2. **OGG protein is targeted to mitochondria.** After transfection and selection, the control and MTS-OGG transfectants were lysed with digitonin, and cell fractions were separated using differential centrifugation. Mitochondrial, nuclear, and cytosolic protein was then analyzed in a Western blot with anti-OGG. The OGG band observed in mitochondria of MTS-OGG cells but not vector controls is ~39 kD. Also shown is a separate portion of the same blot probed with anti-cytochrome C (~15 kD). SDS-Page gels were loaded with 50 µg of protein from: lane 1, MTS-OGG mitochondrial extract; lane 2, control mitochondrial extract; lane 3, MTS-OGG nuclear extract; lane 4, control nuclear extract; lane 5, MTS-OGG cytosolic extract; lane 6, control cytosolic extract.

Fig. 3. Mitochondrial extracts from MTS-OGG transfectants have additional OGG activity. A labeled 24-mer with 8-oxoguanine at the 10th nucleotide (or identical 24-mer without 8-oxoguanine in lanes 4, 6, 8, 10, 12, and 14) was incubated with FPG or cell fraction extracts from MTS-OGG or vector control cells. The reactions contained 0.2 pmol of labeled duplex DNA along with: lane 1, 0.5 U FPG; lane 2, water only; lanes 3 and 4, MTS-OGG mitochondrial extract; lanes 5 and 6, control mitochondrial extract; lanes 7 and 8, MTS-OGG nuclear extract; lanes 9 and 10, control nuclear extract; lanes 11 and 12, MTS-OGG cytosolic extract; lanes 13 and 14, control cytosolic extract. Shown is a representative autoradiograph from four independent experiments.

Fig. 4. Mitochondrial DNA repair of oxidative damage is significantly enhanced in MTS-OGG transfectants. A. Vector only and MTS-OGG transfectants were drugged with 400 μ M menadione for 1 h and either lysed immediately or allowed repair time in their normal media and then lysed. Control samples were exposed to the drug diluent only. Total DNA was isolated from the lysates and analyzed in quantitative alkaline Southern blots with a probe corresponding to part of the human mitochondrial genome. A representative autoradiograph is shown here. B. Solid line – MTS-OGG, and broken line – vector only. Average results +/- S.E.M. from 4 separate repair experiments. An asterisk (*) indicates a significant difference ($p < 0.05$).

Fig. 5. MTS-OGG mitochondria are more functional after oxidative challenge. Cells were treated with 200, 300, 400, and 500 μ M menadione for 1 h and then placed in their normal media. A. After 24 h, the CellTiter 96 assay for reduction of a tetrazolium compound to

formazan was then performed on each sample. B. After 6 h, the ability of the cells to exclude Trypan blue dye was assessed. Shown are average results +/- S.E.M. from 3 separate repair experiments. An asterisk (*) indicates a significant difference ($p < 0.05$).

Fig. 6. **MTS-OGG transfectants survive and multiply to form colonies after oxidative challenge.** Cells were plated sparsely (400 cells per 60 mm dish) and allowed 24 hr to adhere. They were then treated with 25, 50, 75, or 100 μ M menadione for 1 h and then placed in their normal media for 10 days. Colonies were then fixed, stained, and counted. An average of the results +/- S.E.M. from 4 separate clonogenic assays is shown. An asterisk (*) indicates a significant difference ($p < 0.05$).

Figure 1

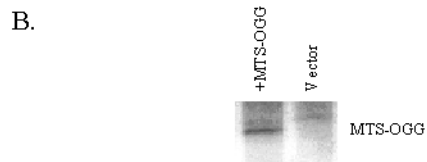
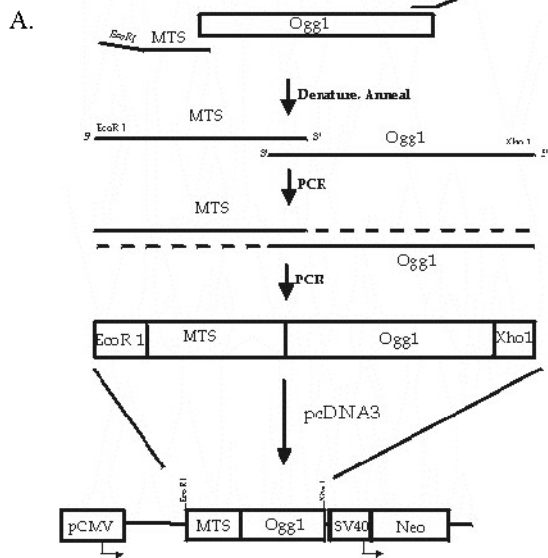


Figure 2

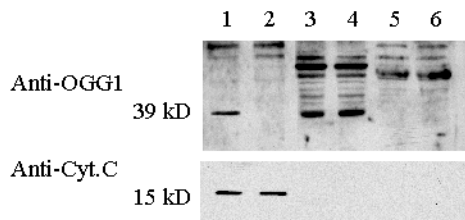


Figure 3

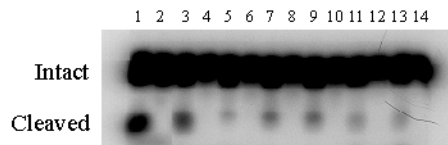
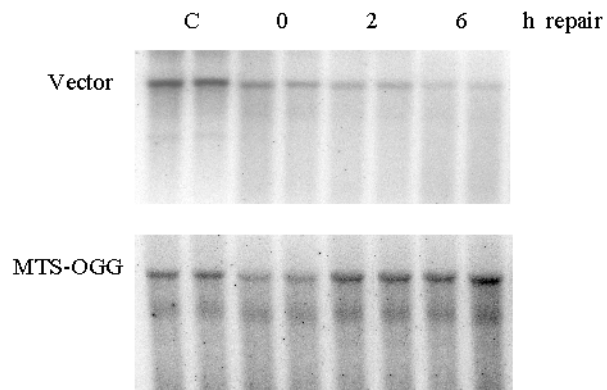


Figure 4

A.



B.

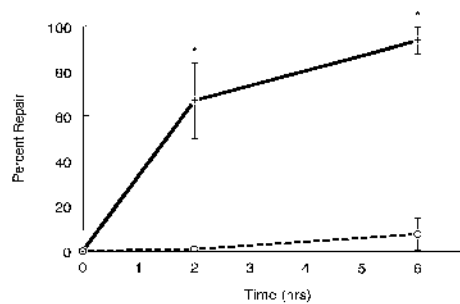
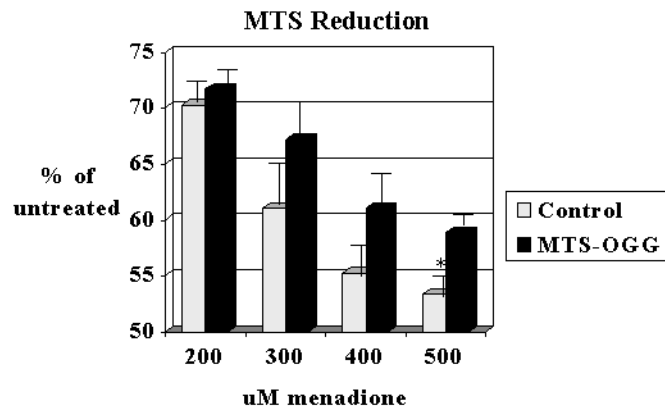


Figure 5

A.



B.

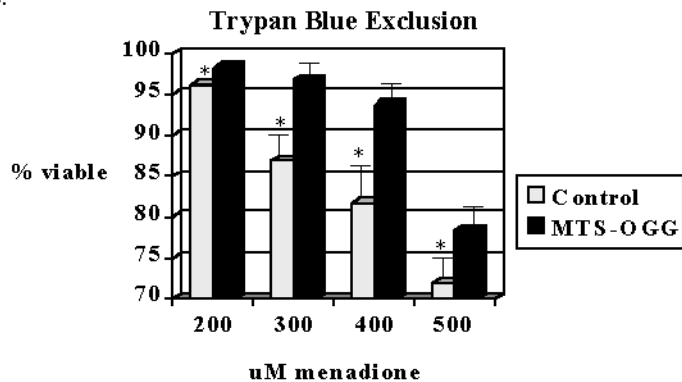


Figure 6

