Repair of Dihydrouracil Supported by Base Excision Repair in mNTH1 Knock-out Cell Extracts*

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In mammalian cells, thymine glycols and other oxidized pyrimidines such as 5,6-dihydrouracil are removed from DNA by the NTH1 protein, a bifunctional DNA-N-glycosylase. However, mNTH1 knock-out mice in common with other DNA glycosylase-deficient mice do not show any severe abnormalities associated with accumulation of DNA damage and mutations. In the present study we used an in vitro repair system to investigate the mechanism for the removal of 5,6-dihydrouracil from DNA by mNTH1-deficient cell-free extracts derived from testes of mNTH1 knock-out mice. We found that these extracts are able to support the removal of 5,6-dihydrouracil from DNA at about 20% of the efficiency of normal extracts. Furthermore, we also found that single-nucleotide patch base excision repair is the major pathway for removal of 5,6-dihydrouracil in mNTH1-deficient cell extracts, suggesting the involvement of other DNA glycosylase(s) in the removal of oxidized pyrimidines.

Reactive oxygen species generate a multitude of different damage to the DNA molecule. In addition to highly cytotoxic double strand breaks, over 20 different types of base damage have been identified in DNA (1). To combat this potentially mutagenic and toxic damage, cells have developed a number of DNA glycosylases that recognize specific types of chemically altered bases and remove them from the DNA in the first step of base excision repair (BER). In mammalian cells, the NTH1 protein, a functional and structural homolog of Escherichia coli endonuclease III, removes oxidized pyrimidines (2, 3). In common with endonuclease III, mNTH1 has a broad substrate specificity, releasing among others thymine glycol, 5,6-dihydrouracil, 5-hydroxycytosine, and ura residues (4).

Recently we reported the generation of a strain of mice deleted in mNTH1 (5). mNTH1 knock-out mice are viable and outwardly normal and show no evident deleterious effects of the glycosylase deletion despite the known mutagenic and toxic properties of the substrate lesions. This finding is similar to that found for other glycosylase-deleted mouse strains, including the mOGG1 knock-out, which lacks glycosylase activity against the major oxidized purine, 8-oxoguanine (6).

Although the reasons for the mild phenotype of the glycosylase-deficient mice are still unclear, evidence is beginning to emerge that indicates that mammalian cells possess other enzymes, both novel glycosylases and damage-specific endonucleases, that can remove the damaged bases in the absence of the deleted enzymes, albeit at a slower rate (7–10). These systems are in addition to alternative mechanisms of repair such as nucleotide excision repair (11) and transcription-coupled repair, which has been implicated in the repair of both 8-oxoguanine and thymine glycol (12, 13). However, there is no direct evidence indicating which repair system is utilized as a backup mechanism in glycosylase knock-out mice.

Thus, in this study we have used cell-free extracts from our mNTH1 knock-out mice in combination with a sensitive DNA repair assay to follow the removal of a unique 5,6-dihydrouracil adduct, one of the major altered bases generated by ionizing radiation under anoxic conditions (14), from closed circular substrate DNA. Our results indicate that short-patch base excision repair is active in removing the modified pyrimidine in extracts derived from both wild-type and mNTH1 knock-out cells.

**Experimental Procedures**

**Materials—**Synthetic oligodeoxyribonucleotides purified by high performance liquid chromatography were obtained from Midland (8-oxoguanine) or Synthegen (5,6-dihydrouracil). [γ-32P]ATP (3000 Ci/mmol) was purchased from PerkinElmer Life Sciences.

**NTH1+/− Mice—**The generation and initial characterization of our NTH1+/− mice will be described in detail elsewhere; however, brief details are included here for clarity. The mNth1 gene consists of 6 exons and is immediately adjacent to the tuberous sclerosis 2 gene on chromosome 17 (2). Our targeting vector consisted of a pgk-neo cassette inserted between an Eco47III and AvaI site in exon 4, resulting in the deletion of 65 base pairs and disrupting the conserved helix-hairpin-helix motif that is involved in substrate binding (15). Homologous recombination in embryonic stem cells was confirmed by long range PCR and Southern blotting, and correctly targeted cells were microinjected into C57Bl/6J mouse blastocysts. NTH1+/− mice were obtained by crossing the NTH1+/− offspring of the resulting chimeras.

**Proteins—**Histidine-tagged human AP endonuclease 1 was purified on Ni2+−charged His-Bind Resin (Novagen, Cambridge, MA) as recommended by the manufacturer.

**DNA Substrates—**The oligonucleotides 5′-ATATACGCGG[C/A]G-GCGTAACGCTATT-3′ (30 pmol) and 5′-ATATACGGGGCUAGCAGCTATT-3′ (where U stands for dihydrouracil, 30 pmol) were 5′-end-labeled with 100 μCi (33 pmol) of [γ-32P]ATP and used for construction of substrates containing single 8-oxoguanine or dihydrouracil in circular closed double-stranded DNA as described previously (16).

**BER Reactions—**The BER reactions were carried out as described previously (17). In brief, the reaction mixture (50 μl) contained 50 mM Hepes-KOH, pH 7.8, 50 mM KC1, 10 mM MgCl2, 0.5 mM EDTA, 1.5 mM dithiothreitol, 2 mM ATP, 0.4 mg/ml bovine serum albumin, 25 mM phosphocreatine (di-Tris salt, Sigma), 2.5 μg of creatine phosphokinase

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Results

Repair of 8-Oxoguanine Is Efficient in mNTH1 Knock-out Cells—Two different cell extracts, prepared from testes collected from normal or mNTH1 knock-out mice, were used in this study. To demonstrate that these extracts are equally active in DNA repair of a specific lesion other than dihydrouracil, we used closed circular double-stranded DNA substrates bearing a single 8-oxoguanine/cytosine base pair at a defined position (Fig. 1A). The damage-containing strand of the substrate was 32P-labeled upstream of the damage site. HindIII cleavage of the DNA substrates released a 59-mer labeled fragment containing the damage (Fig. 1B, lanes 1 and 2). This fragment has two HaeIII restriction sites, but one of them is blocked by 8-oxoguanine (17), and thus simultaneous cleavage with HaeIII and HindIII restriction endonucleases of unrepaired substrate would generate a 52-mer labeled product containing 8-oxoguanine (unrepaired fragment). Following repair of the 8-oxoguanine-containing substrate the second HaeIII site will be restored and should give rise to a 48-mer (repaired fragment).

The 8-oxoguanine-containing substrate DNA was incubated with whole-cell extract for 1 h, and after isolation from the reaction mixture it was split into two equal aliquots. One-half of the sample was then treated with HindIII, while the DNA in the other was subjected to simultaneous cleavage with HindIII and HaeIII. Thus, after HindIII cleavage we observed only the release of a 59-mer labeled product (Fig. 1B, lanes 1 and 2) and did not find accumulation of any repair intermediates. However, simultaneous cleavage with HindIII and HaeIII indicated that 85–90% of the 8-oxoguanine was efficiently repaired by both extracts (Fig. 1B, lanes 3 and 4, 48-mer).

Repair of Dihydrouracil by Normal and mNTH1 Knock-out Cell Extracts—As with the 8-oxoguanine-containing substrate, in the dihydrouracil-containing substrate the HpaII restriction site is blocked by dihydrouracil (17), and thus simultaneous cleavage of unrepaired substrate DNA with HpaII and HindIII restriction endonucleases generates only a 59-mer labeled product containing dihydrouracil. However, once repair of the dihydrouracil-containing substrate has taken place, HindIII-HpaII cleavage will generate a 49-mer fragment (Fig. 2A).

Repair of the dihydrouracil-containing substrate by testes cell extracts from normal mice was significantly slower than the repair of 8-oxoguanine, and preliminary experiments indicated that at least 3 h are required for processing of ~30% of the substrate (data not shown). Thus, the dihydrouracil-containing substrate DNA was incubated with testes cell extracts from normal or mNTH1 knock-out mice for 3 h at 37 °C. Following recovery from the reaction mixture, it was split into two equal aliquots, and one-half of the sample was then treated with HindIII (lanes 1 and 2) or HindIII/HpaII (lanes 3 and 4). Reaction products were analyzed by 10% denaturing polyacrylamide gel electrophoresis.

FIG. 1. Repair of 8-oxoguanine-containing substrate by normal and mNTH1-deficient cell extracts. A, schematic presentation of 8-oxoguanine-containing substrate. The sites of cleavage by the restriction enzymes HindIII and HaeIII and the position of the 32P label are shown. The site of incision by AP endonuclease subsequent to glycosylase processing of 8-oxoguanine (8-oxo) is indicated with an arrow. B, repair of 8-oxoguanine by whole-cell extracts. Reactions contained 50 ng of substrate DNA and 100 μg of whole-cell extract derived from normal (lanes 1 and 3) or mNTH1 knock-out mouse testes (lanes 2 and 4). Reactions were incubated at 37 °C for 1 h prior to isolation of the substrate DNA followed by digestion with HindIII (lanes 1 and 2) or HindIII/HaeIII (lanes 3 and 4). Reaction products were analyzed by 10% denaturing polyacrylamide gel electrophoresis.

FIG. 2. Repair of dihydrouracil-containing substrate. A, schematic presentation of dihydrouracil-containing substrate. The sites of cleavage by the restriction enzymes HindIII and HpaII and the position of the 32P label are shown. The site of incision by AP endonuclease subsequent to glycosylase processing of dihydrouracil is indicated with an arrow. DHU, dihydrouracil. B, repair of dihydrouracil by testes whole-cell extract. Reactions contained 50 ng of substrate DNA and 100 μg of whole-cell extract derived from normal mouse testes (lanes 1 and 3) and from mNTH1 knock-out mouse testes (lanes 2 and 4). Reactions were incubated at 37 °C for 1 h prior to isolation of the substrate DNA followed by digestion with HindIII (lanes 1 and 2) or HindIII/HpaII (lanes 3 and 4). Reaction products were analyzed by 10% denaturing polyacrylamide gel electrophoresis.
representing the repaired fraction is due to contamination of the substrate DNA with AP sites or may have been generated by cleavage of unrepairable substrate with HpaII. Unrepairable substrate DNA was treated with purified human AP endonuclease and cleaved with HindIII (Fig. 3, lane 2) or with HpaII (Fig. 3, lane 3) followed by HindIII cleavage. Even after substantial overexposure of the gels, we did not find any significant amount of 49-mer product. We thus conclude that the 49-mer restriction product observed after incubation with either cell extract indeed represented the repaired substrate.

**BER Is the Major Pathway for Dihydrouracil Repair in mNTH1 Knock-out Cell Extracts**—We have previously demonstrated that BER is the major repair pathway for removal of oxidized pyrimidines (19). However, in the absence of mNTH1, the major DNA glycosylase responsible for removal of dihydrouracil in DNA, there is a possibility for increased involvement of other repair systems, namely nucleotide excision repair (NER) (11, 20, 21) and nucleotide incision repair (7), in processing of dihydrouracil. During NER, the damaged base is excised as part of an oligonucleotide that includes 20–24 nucleotides 5′ to five to nine nucleotides 3′ to the damaged site (22, 23). Since the dihydrouracil-containing substrate was labeled 12 nucleotides 5′ to the damage, 25–30-mer labeled oligonucleotides should be generated as a result of NER. However, we did not see 25–30-mer products even after substantial overexposure of the gels (Fig. 3, lane 4).

The BER pathway proceeds mainly through a single-nucleotide replacement mechanism (24). In contrast, the mechanism proposed for nucleotide incision repair involves at least a two-nucleotide repair patch (7). To evaluate the role of nucleotide incision repair in repair of dihydrouracil in mNTH1 knock-out cell extracts we changed the repair reaction conditions so that only short-patch repair was allowed (a mixture of dCTP and ddGTP was used instead of all four dNTPs). Under these conditions any extension of the repair gap beyond one nucleotide will lead to incorporation of ddGMP (see Fig. 2A) and termination of both DNA synthesis and ligation, detectable by accumulation of incised, but unligated, products after HindIII hydrolysis. As for human cell extracts (16, 25), repair in wild-type mouse testes extracts was mostly accomplished through insertion of a single nucleotide (Fig. 4, lane 3, 49-mer product). Only a small amount proceeded via a synthesis of a repair patch longer than one nucleotide (long-patch pathway), which results in a repair block and accumulation of some 51-mer product.

**DISCUSSION**

The absence of any clearly discernable, detrimental phenotype in our mNTH1−/− mice is highly suggestive of the action of one or more compensatory repair pathways that remove potentially toxic pyrimidine bases from damaged DNA. The results presented in this article indicate that, for dihydrouracil at least, the principal method of repair in the absence of mNTH1 is by one-nucleotide gap filling by the base excision repair pathway. Extracts from mNTH1−/− mice were equally proficient at directing the repair of 8-oxoguanine (Fig. 1) indicating that the activity of 8-oxoguanine-DNA glycosylase was unaffected by the deletion of mNTH1 protein and that both extracts were capable of carrying out the BER reaction. However as expected, differential repair of dihydrouracil was observed when this DNA adduct was substituted for 8-oxoguanine. Nevertheless, although significantly reduced, repair of this oxidized pyrimidine was observed when mNTH1−/− extracts were used in the reaction.

During the preparation of this article, two groups (8, 10) reported the initial characterization of two novel bifunctional human DNA glycosylases, NEH1/NEIL1 and NEH2/NEIL2, with homology to the E. coli MutM (Fpg) and Nei (endonuclease VIII) proteins. Based on the limited information available to date, it is likely that NEIL1 is more active on duplex oligonucleotides containing a single dihydrouracil, although it is interesting to note that incubation of NEIL1 with γ-irradiated calf thymus DNA did not result in the release of dihydrouracil (8). Therefore, it is possible that the initiation of the BER activity observed in our assays is due to NEIL1 or NEIL2.

However, using extracts from a different mNTH1−/− mouse strain, Takao et al. (9) have recently described the partial characterization of two novel thymine glycol-DNA glycosylases (TGG1 and TGG2). These enzymes can be identified by their different reaction mechanisms and are different again from the human NEIL homologs. Thus, while NEIL1 and NEIL2 possess a ββ-elimination activity, TGG1 is most likely a monofunctional glycosylase unable to carry out strand incision, and TGG2 resembles E. coli endonuclease III (Nth) in its mode of action, achieving strand nicking by β-elimination. Therefore, it will be interesting to learn whether the method by which strand scission is achieved ultimately determines the mechanism of gap filling. Further characterization of the substrate specificities of these enzymes is required to determine whether dihydrouracil is also a substrate for either of the TGG enzymes.

From our results, the compensatory pathways are unlikely to be due to NER or the recently described nucleotide incision
repair pathway. We found no evidence of NER incision products in our assays (Fig. 3), while under polymerase blocking conditions, there was no reduction in intensity of the 49-mer products in our assays (Fig. 3). This result effectively rules out incision of the dihydrouracil by a damage-specific endonuclease, which would give rise to a dangling nucleotide at the 5’ terminus and repair through the long-patch BER pathway (7).

In conclusion, the availability of mammalian cell-free extracts lacking mNTH1 has enabled us to identify a novel BER activity for the removal of dihydrouracil. Recent results from our own laboratory (5) and others (8–10) are revealing the presence of multiple, hitherto unknown, DNA glycosylases and other damage-sensing enzymes. We are currently continuing our studies to determine the nature of the glycosylase(s) responsible for the repair activity described here to fully characterize its mode of action and substrate specificities.

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