

Efficient *in vitro* repair of 7-hydro-8-oxodeoxyguanosine by human cell extracts: involvement of multiple pathways

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ABSTRACT

To investigate the repair of oxidative damage in DNA, we have established an *in vitro* assay utilizing human lymphoblastoid whole cell extracts and plasmid DNA damaged by exposure to methylene blue and visible light. This treatment has been shown to produce predominantly 7-hydro-8-oxodeoxyguanosine (8-oxodG) in double-stranded DNA at low levels of modification. DNA containing 1.6 lesions per plasmid is substrate for efficient repair synthesis by cell extracts. The incorporation of dGMP is 2.7 ± 0.5 times greater than the incorporation of dCMP, indicating an average repair patch of 3–4 nucleotides. Damage-specific nicking occurs within 15 min, while resynthesis is slower. The incorporation of dGMP increases linearly, while the incorporation of dCMP exhibits a distinct lag. Extracts from *xeroderma pigmentosum* (XP) complementation groups A and B exhibit 25 and 40%, respectively, of the incorporation of dCMP compared with normal extracts, but extracts from an XP-D cell line exhibit twice the activity. These data suggest that the efficient repair of 8-oxodG lesions observed in human cell extracts involves more than one pathway of base excision repair.

INTRODUCTION

Oxidative damage to DNA is an inevitable consequence of metabolic activity and the chemical instability of DNA (1–3). Oxidative damage in DNA has often been suggested as a contributing factor in the process of aging and the development of cancer (4). The repair of oxidative damage in DNA by human cells is not well understood due to the multitude of lesions produced by oxidative treatments (5) and the complexity of the response. The repair of damage induced by ionizing radiation and other agents producing base modifications as well as single-strand breaks has both aphidicolin-sensitive and -resistant components (6), which has been interpreted in terms of long patch and short

patch repair (7). In permeabilized human fibroblasts, the repair of damage induced by the radiomimetic agent bleomycin involves short patch repair with polymerase β and long patch repair with polymerase δ (8). However, because the above treatments produce a variety of lesions in DNA, heterogeneity in the repair has been attributed to the repair of different lesions by different repair pathways.

One of the most studied and important lesions produced in DNA by reactive oxygen species is 7-hydro-8-oxodeoxyguanosine (8-oxodG). Due to its mispairing with deoxyadenosine, 8-oxodG is mutagenic (9,10). In *Escherichia coli*, 8-oxodG is removed from DNA by either a specific glycosylase, known as formamidopyrimidine glycosylase (Fpg protein), in the initial step of base excision repair (BER), or by the UvrABC enzymes through nucleotide excision repair (NER) (11). Cells deficient in either pathway do not show an appreciable defect in the repair of 8-oxodG, but the double mutants exhibit sensitivity to agents producing 8-oxodG lesions (11). Eukaryotic cells may also employ multiple pathways for the removal of this common lesion. Separable activities of an 8-oxodG endonuclease and an 8-oxodG-specific glycosylase have been identified in human cells (12). The yeast (13,14), mouse (15) and human (16,17) genes encoding homologous 8-oxodG-specific glycosylase/apurinic lyases have recently been cloned. A similar mitochondrial-specific activity has also been characterized (18). The *Drosophila* ribosomal protein S3 displays specific 8-oxodG and AP activities (19). The human, but not the mouse, *N*-methylpurine DNA glycosylase exhibits an 8-oxodG incising activity (20). In addition, the involvement of the human NER system in the repair of 8-oxodG has recently been demonstrated (21). However, the relative importance of these activities in the overall repair of 8-oxodG in human cells has not been evaluated.

Most agents producing oxidative damage in DNA result in the formation of multiple DNA base lesions. However, photoactivated methylene blue produces almost exclusively the base modification 8-oxoguanosine in double-stranded DNA (22,23) without significant conversion of the other three bases (24). With 1% of the guanosines modified, 8-oxoguanosine and formamidopyrimidine are detected in a ratio of 20:1 (23). At twice that level of

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modification, an additional product is detected, but at <0.3% of the level of 8-oxodG (25). At higher levels of modification, eight additional species are formed (24), although 8-oxodG remains the most prevalent. In addition to 8-oxodG, the formation of another lesion in single-stranded DNA, but not double-stranded DNA, has been proposed based on the mutation spectrum (26). Thus under conditions in which <1% of the guanines are modified, the reaction of double-stranded DNA with photoactivated methylene blue produces predominantly 8-oxodG.

Although the presence of 8-oxodG-specific glycosylases in mammalian cells has been amply demonstrated, little is known about the overall repair of this lesion. DNA damaged by exposure to methylene blue and visible light represents a useful model system for *in vitro* investigation of the repair of a common oxidative lesion in DNA due to the near-exclusive formation of the lesion 8-oxodG. We have used plasmid DNA damaged by methylene blue and visible light to investigate the repair of 8-oxodG by extracts from human cells.

MATERIALS AND METHODS

Materials

Radioisotopes [α - 32 P]dCTP and [α - 32 P]dGTP (3000 Ci/mmol) were purchased from Amersham or DuPont NEN Research Products. Radiolabeled dGTP was used within 5 days of the reference date. Methylene blue was obtained from Ricca Chemical. ATP, dNTPs and restriction enzymes were obtained from Boehringer Mannheim. Aphidicolin, HEPES, creatine phosphokinase (rabbit muscle), phosphocreatine and protease inhibitors were purchased from Sigma. BSA, cell culture media and supplements were purchased from GIBCO BRL. Fpg enzyme (5×10^7 U/mg) was obtained from Dr A.P.Grollman (SUNY, Stony Brook, NY). Endonuclease III was purchased from Trevigen Inc.

Plasmid DNA

pBluescript II KS(+) (Stratagene), referred to herein as pKS(+), and pRS, a 4470 bp derivative of pUC19 constructed by Dr G.Dianov (NIA, Baltimore MD), were propagated in XL1-Blue cells (Stratagene) and prepared by the lysozyme-Triton X-100 method (27) with modifications. Phenol and chloroform were not used during plasmid purification. pKS(+) DNA was purified by cesium chloride-ethidium bromide equilibrium centrifugation prior to treatment with damaging agents. Undamaged pRS DNA was purified by a total of three cesium chloride-ethidium bromide centrifugation steps and one sucrose gradient sedimentation step.

UV-damaged pKS(+) plasmid was prepared by irradiating DNA at 50 μ g/ml in 40 mM HEPES-KOH (pH 7.9), 0.1 M KCl, 0.5 mM EDTA with UV-C (254 nm) at a fluence of 1.01 W/m² for a total of 450 J/m². The irradiated plasmid was treated with *E.coli* endonuclease III to remove pyrimidine hydrates and further purified by two cesium chloride-ethidium bromide gradients and one sucrose gradient. The resulting plasmid is free of nicks and serves as a standard substrate for NER activity.

Oxidatively damaged pKS(+) was prepared by treating 0.1 mg/ml DNA with 10 μ M methylene blue, in 0.01 M sodium phosphate buffer (pH 7.4). The plasmid DNA was kept on ice and exposed to visible light at a fluence of 117 W/m², from a 100 W tungsten bulb, for varying amounts of time. In addition, pKS(+) DNA treated with methylene blue but kept in the dark was prepared as

an external control. Methylene blue was removed by three consecutive ethanol precipitation steps. Except for the visible light exposure, all manipulations with methylene blue were performed in the dark or under a dim blue safelight. Plasmid DNA was further purified by two cesium chloride-ethidium bromide gradient centrifugation steps and one sucrose gradient step. None of the plasmids had a detectable population of nicked molecules and the fraction of nicked molecules did not increase significantly during storage at 4°C for 4 months.

Preparation of whole cell extracts

Human lymphoblastoid cell lines were obtained from NIGMS Human Genetic Mutant Cell Repository and NIA Aging Cell Repository (Coriell Institute for Medical Research, Camden, NJ). The cell lines were: GM01310b (normal), AG09387 (normal), GM02250e (XPA), GM02252a (XPB), GM02253e (XPD), GM01857 (CSA) and GM01712a (CSB). Cells were grown in RPMI 1640 media, supplemented with 13% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin G and 100 μ g/ml streptomycin sulfate, and maintained at 37°C with 5% CO₂ and 95% humidity. All cell lines were negative for mycoplasma contamination. The cells were harvested at a density of $\sim 6 \times 10^5$ cells/ml. Whole cell extracts (WCE) were prepared as described (28). WCE exhibited the expected proficiency or deficiency for repair incorporation into UV-damaged plasmid and XP WCE exhibited the expected pattern of complementation (29).

Fpg nicking assay

DNA damaged by exposure to methylene blue and visible light was treated with Fpg protein to determine the number of enzyme-sensitive sites. DNA at a concentration of 2.5 μ g/ml in 50 mM Tris-HCl (pH 8.0), 50 mM KCl and 1 mM EDTA was incubated with various amounts of Fpg protein in a 20 μ l volume at 37°C for 25 min. The products were separated on a 1% agarose gel containing 0.5 μ g/ml ethidium bromide. The relative fluorescence of the supercoiled and nicked bands was determined using a FluorImager (Molecular Dynamics) and analyzed using Image-Quant software (Molecular Dynamics). Assuming a Poisson distribution of the damage, the average number of enzyme sensitive sites per plasmid is given by the expression: $-\ln(1.4 \times F_s / (1.4 \times F_s + F_n))$, where F_s and F_n are the fluorescence of the supercoiled and nicked bands, respectively, and the factor 1.4 corrects for the difference in ethidium bromide binding to supercoiled compared to linear DNA.

Repair incorporation assay

Repair incorporation assays were performed essentially as described (28). Briefly, reactions contained 300 ng each of supercoiled damaged pKS(+) and undamaged pRS, 100 μ g of WCE protein in a volume of 50 μ l in a solution of 44 mM HEPES-KOH (pH 7.9), 70 mM KCl, 7.5 mM MgCl₂, 1.2 mM DDT, 0.5 mM EDTA, 2 mM ATP, 250 μ g/ml BSA, 40 mM phosphocreatine, 50 μ g/ml creatine phosphokinase, and 50 μ M each of dATP and dTTP. In addition, reactions contained either 50 μ M dGTP, 5 μ M dCTP and 1 μ Ci of [α - 32 P]dCTP or 50 μ M dCTP, 5 μ M dGTP and 1 μ Ci of [α - 32 P]dGTP. The reactions were incubated at 30°C for 2 h, stopped by addition of EDTA, and then treated with RNaseA followed by proteinase K. The DNA was recovered by phenol-chloroform extraction and ethanol precipitation. Plasmids were linearized with

EcoRI endonuclease and separated by electrophoresis through a 1% agarose gel, containing 0.5 $\mu\text{g/ml}$ ethidium bromide. The gel was scanned to measure the intensity of ethidium bromide fluorescence (FluorImager, Molecular Dynamics). The gel was dried under vacuum and exposed to a storage screen (PhosphorImager, Molecular Dynamics) for 41–42 h.

Kinetics experiments

Reactions to determine the rate of nucleotide incorporation were performed similarly, except that the reaction mix containing cell extracts was preincubated at 30°C for 5 min before the reaction was initiated by addition of DNA. WCE nicking experiments were performed similarly, except that dNTPs were omitted from the reaction and the DNA was not linearized.

Data analysis

Autoradiograms and images of ethidium bromide fluorescence were analyzed using ImageQuant software (Molecular Dynamics). Gel image intensities are displayed with a linear response to the signal. Fluorescence intensities of linearized pKS(+) and pRS bands, in comparison with known amounts of DNA, were used to determine the DNA loading. Background-corrected values of the radioactivity incorporated for the damaged and undamaged plasmids were normalized for the amount of DNA. The damage-specific radioactivity incorporated was calculated by subtracting the background-corrected and normalized radioactivity incorporated in undamaged pRS from that incorporated into damaged pKS(+). In calculating the moles of nucleotide incorporated, the amount of ^{32}P -labeled nucleotide was corrected for decay. The counts per minute of radioactivity incorporated may be related to the moles of the specific nucleotide incorporated by the use of the equation:

$$\text{moles } dNMP = \frac{(\text{c.p.m. incorporated})(5 \mu\text{M})(50 \mu\text{l})}{(\text{counting efficiency})(2.2 \times 10^6 \text{ d.p.m.})(\text{decay factor})} \quad 1$$

The radioactivity incorporated (d.p.m.) was determined by liquid scintillation counting of the excised band or by using a particular phosphorus storage screen for which the counting efficiency had been determined.

Analysis of the ratio of dGMP to dCMP incorporation by DNA repair pathways

Assuming that the damage is localized exclusively to guanosine and that the surrounding sequence does not affect the extent of damage formation, then the average size of the repair patch can be deduced from the differential incorporation of dGMP to dCMP. We analyzed the sequence of pKS(+) (GenBank #X52327) using a custom program written in Visual Basic. The fraction of all Gs having a G immediately 3' was $q_{2,G} = 0.257$ while the fraction with a C in that position was $q_{2,C} = 0.300$. Similarly for the next position to the 3', $q_{3,G} = 0.279$ and $q_{3,C} = 0.216$, and so on. Averaging up to the patch size, n , leads to an expression for the ratio of dGMP to dCMP incorporated,

$$r = \frac{\sum_{i=1}^n q_{i,G}}{\sum_{i=1}^n q_{i,C}} \quad 2$$

For patch sizes of 2–6, the ratio of dGMP to dCMP incorporated is predicted to take the values: 4.19, 2.98, 2.35, 2.03, 1.78.

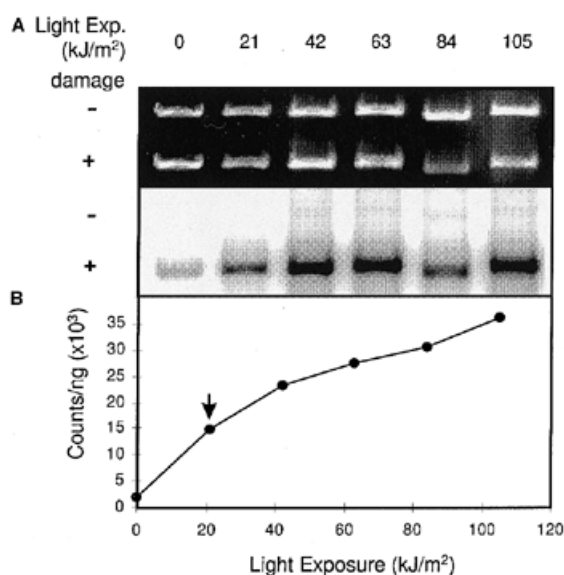


Figure 1. Increasing light exposure to methylene-blue treated DNA increases *in vitro* repair incorporation by human lymphoblast cell extracts. Standard reactions containing WCE from GM01310b (normal) cells, undamaged DNA (– damage) and DNA treated with 10 μM methylene blue and exposed to various amounts of visible light (+ damage). (A) Gel images show ethidium bromide staining of DNA and incorporation of [α - ^{32}P]dCMP. (B) Damage-specific incorporation of [α - ^{32}P]dCMP is expressed as counts per ng and increases with increasing exposure to light. The arrow marks the exposure used for subsequent experiments. This experiment was repeated with similar results but the values were not averaged because PhosphorImager screens with different efficiency were used.

However, if repair proceeds by two pathways, one replacing only the modified base and a second replacing the modified G and a characteristic number of adjacent nucleotides, then the proportion of the lesions that are repaired by each pathway can be determined if the patch size of the second pathway, m , is known. The fraction of the lesions repaired by the second pathway, f_b is given by the expression:

$$f_b = 1 / (1 + r_{\text{obs}} \cdot \sum_{i=1}^m q_{i,C} - \sum_{i=1}^m q_{i,G}) \quad 3$$

where r_{obs} is the observed ratio of dGMP to dCMP incorporated. We have considered the second pathway to be either ‘short patch’ BER with an average patch size of $m = 6$, or NER with a patch size of ~ 28 (30). The assumption of the patch size of 6 is at the upper end of the range usually given for the ‘short patch’ BER pathway, so this calculation may underestimate the contribution of that pathway.

RESULTS

Effect of increased dose of visible light during methylene-blue treatment of DNA on *in vitro* repair synthesis by normal WCE

As shown in Figure 1, DNA repair synthesis experiments with normal human WCE contain both undamaged and oxidatively damaged plasmids. The incorporation of [α - ^{32}P]dCMP into the methylene blue-treated DNA (+ damage) increased with light exposure from 0 to 105 kJ/m^2 and is highly specific for damage

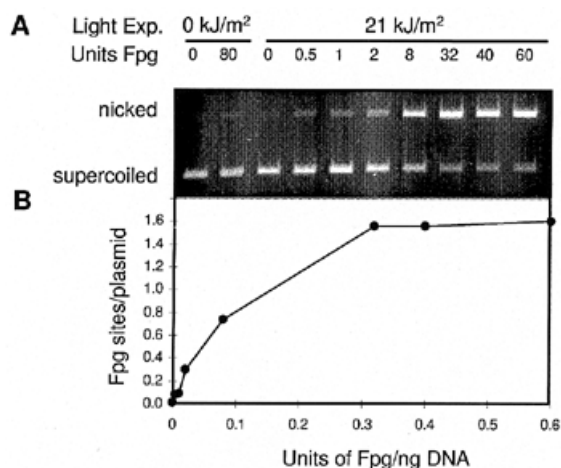


Figure 2. Determination of the number of Fpg glycosylase-sensitive sites in pKS(+) DNA treated with methylene blue and 21 kJ/m² of visible light. Ethidium bromide stained gel showing conversion of supercoiled to nicked DNA by Fpg protein. The number of enzyme sensitive sites per plasmid was determined using the Poisson distribution.

generated by the combination of methylene blue and visible light. Very little radioactivity is incorporated into the undamaged plasmid (– damage). The lowest dose of visible light (indicated by an arrow) produced significant damage-specific incorporation and was used as the source of 8-oxodG-containing DNA in subsequent experiments. The damage-specific incorporation into the control DNA, which was exposed to methylene blue but not irradiated, was 7–20 times lower than the incorporation into DNA exposed to methylene blue and 21 kJ/m² of visible light. Although the incorporation of radioactivity into the external control DNA is low in comparison with the irradiated DNA, it is significantly greater than the incorporation into the undamaged control DNA (pRS).

Determination of the number of Fpg protein-sensitive sites

Fpg protein possesses 8-oxodG glycosylase and AP lyase activities (23,31). We determined the number of Fpg protein-sensitive sites per plasmid molecule created by exposing the pKS(+) plasmid to 21 kJ/m² visible light in the presence of 10 μM methylene blue. As shown in Figure 2, the fraction of the plasmid DNA nicked by Fpg protein approached a limiting value and indicated the presence of 1.6 Fpg protein-sensitive sites per plasmid. The methylene blue treated but unexposed plasmid yielded ~0.07 enzyme sensitive sites per plasmid. Fpg protein also recognizes formamidopyrimidine (23,31).

Efficiency of repair incorporation by cell extracts into methylene blue and UV-damaged DNA

The incorporation of [α-³²P]dCMP by normal WCE into the methylene blue-damaged plasmid and the UV-damaged plasmid is shown in Figure 3. Four-fold more dCMP was incorporated into the oxidatively damaged plasmid than into the UV-irradiated plasmid at these levels of DNA modification. The greater efficiency of repair is more striking when the number of sites is considered. UV-C (254 nm) irradiation of 450 J/m² produces ~10 cyclobutane pyrimidine dimers and about three [6-4] photoproduct lesions per similarly-sized plasmid molecule (28). Pyrimidine hydrates are also formed

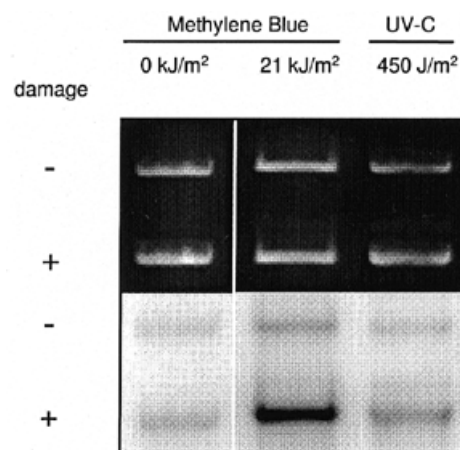


Figure 3. Efficient DNA repair synthesis into DNA containing methylene blue and visible light-induced damage compared with UV-damaged DNA. Gel images show ethidium bromide staining of DNA and incorporation of [α-³²P]dCMP. The larger plasmid (– damage) is the undamaged internal control. WCE from normal human cells (GM01310b) incorporate [α-³²P]dCMP into DNA damaged by methylene blue and 21 kJ/m² of visible light more efficiently than into DNA damaged by 450 J/m² UVC.

by the UV-C irradiation (28) but were removed by the subsequent treatment by endonuclease III followed by cesium chloride–ethidium bromide banding. Since cyclobutane pyrimidine dimers are poorly recognized by the NER enzymes, it has been reported that 75% of the damage-specific repair synthesis occurs at the [6-4] photoproducts (28). As shown above, exposing pKS(+) DNA to methylene blue and 21 kJ/m² of visible light produces 1.6 Fpg protein-sensitive sites per plasmid. Thus the incorporation of dCMP by the normal cell extracts is considerably more efficient per 8-oxodG than per [6-4] photoproduct.

Specific nucleotide differences in DNA repair incorporation into methylene blue-damaged DNA by human whole cell extracts

The amount of radioactivity incorporated by human cell extracts during the repair of methylene blue-induced damage depends on the identity of the labeled nucleotide. As shown in Figure 4, approximately equal amounts of [α-³²P]dGMP and [α-³²P]dCMP are incorporated into the methylene blue damaged plasmid, although the amount of DNA loaded in the dCMP lane is approximately three times greater. Based on several experiments, the specific incorporation of [α-³²P]dGMP into the oxidatively damaged DNA is 2.7 ± 0.5 times greater than the incorporation of [α-³²P]dCMP. Little of either nucleotide is incorporated into the larger, undamaged plasmid also present in the reaction. Considering the specificity of photoactivated methylene blue for producing 8-oxodG in double-stranded DNA (23,25), the relatively small ratio of dGMP to dCMP incorporation during *in vitro* repair by human WCE indicates that a significant component of the repair replaces adjacent nucleotides in addition to the modified guanosine.

We have performed DNA repair synthesis experiments with the concentration of the limiting nucleotide varied between 2 and 20 μM and the amount of labeled nucleotide constant at 1 μCi (data not shown). The amount of nucleotide incorporated, when calculated according to equation 1 and corrected for isotopic

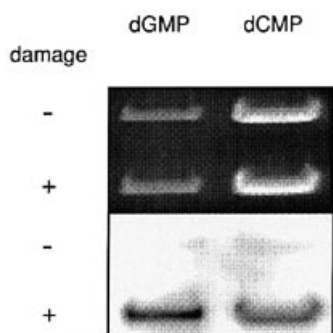


Figure 4. Nucleotide-specific incorporation by normal human whole cell extracts (GM01310b) into DNA damaged by exposure to methylene blue and visible light. Gel images show ethidium bromide staining of DNA and incorporation of [α - 32 P]dGMP or [α - 32 P]dCMP into damaged or undamaged DNA. The larger plasmid (- damage) was an untreated internal control. The smaller plasmid (+ damage) was treated with 10 μ M methylene blue and 21 kJ/m 2 of visible light.

dilution, is essentially constant over this range. Thus residual nucleotides in the cell extracts do not contribute significantly in the reaction. In addition, experiments with radiolabeled nucleotide used immediately after delivery or 10 days later give the same ratio of incorporated dGMP to dCMP (data not shown), indicating that degradation products in the labeled nucleotide stock do not significantly affect incorporation.

Kinetics of repair of methylene blue induced damage

To determine the rate limiting step in the repair of 8-oxodG, the kinetics of nicking and resynthesis by the WCE was investigated. Damage specific nicking of the methylene blue-damaged plasmid by the WCE occurred rapidly, with a limiting value of \sim 0.5 nicks per plasmid being attained within 15 min (data not shown). The undamaged plasmid, pRS, was also nicked by the WCE, exhibiting \sim 0.07 non-specific nicks per 3 kb in that time.

In contrast, the resynthesis step is slower. As shown in Figure 5, the incorporation of radiolabeled dCMP or dGMP increases with time for incubations up to 2 h. Interestingly, the incorporation of dCMP exhibits a distinct lag, with almost negligible incorporation until after 15 min of incubation. Together, these results suggest that incision is not rate limiting for the repair of 8-oxodG and that some portion of the repair pathway detected by the incorporation of dGMP may differ from that detected by the incorporation of dCMP.

Effect of DNA polymerase inhibitors on incorporation of radiolabeled dGMP and dCMP by normal human WCE into methylene blue-damaged DNA

The effects of specific DNA polymerase inhibitors were examined and the results are summarized in Table 1. The incorporation of dCTP is moderately sensitive to the presence of aphidicolin, exhibiting 58% of the activity compared to the control. Addition of ddTTP alone has no effect on the incorporation, but in the presence of both inhibitors, the activity is reduced to 33%. The incorporation of dGTP is comparatively less sensitive to aphidicolin and more sensitive to ddTTP, giving 94 and 77% of the activity,

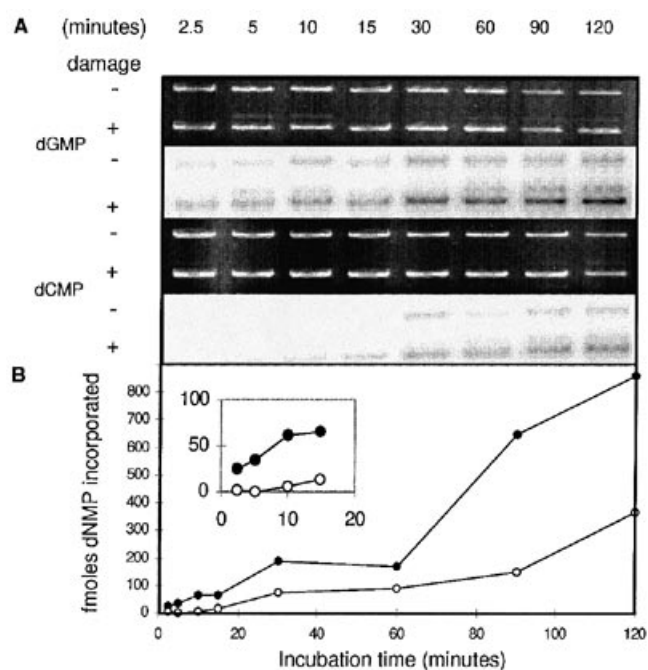


Figure 5. Kinetics of nucleotide incorporation by GM01310 (normal) WCE into DNA damaged by methylene blue and visible light. (A) Gel images showing DNA loading and incorporation of radioactivity for reactions containing [α - 32 P]CTP and [α - 32 P]GTP. (B) Time-dependence of damage specific incorporation of radioactivity. Incorporation of radioactivity was determined by liquid scintillation counting of excised bands and is expressed as fmoles of dNMP incorporated per 300 ng of damaged DNA: closed circles, [α - 32 P]GTP; open circles, [α - 32 P]CTP.

respectively. In the presence of both inhibitors, the incorporation of dGTP is reduced to 45% of the control.

With purified proteins, aphidicolin and ddTTP are selective inhibitors of pol δ (or pol ϵ) and pol β , respectively. However, whole cell extracts or permeabilized cells have often displayed only partial inhibition (30,32,33). Although the inhibitors are only partially effective in the present system, the differences in the sensitivities of dGMP and dCMP incorporation to the inhibitors suggests differences in the repair pathways detected by the two nucleotides.

Table 1. Effects of DNA polymerase inhibitors on the incorporation of radiolabeled dCMP or dGMP into methylene blue-damaged DNA by normal whole cell extracts

Inhibitor	fmoles dCMP incorporated ^a	relative dCMP incorporation	fmoles dGMP incorporated ^a	relative dGMP incorporation
none	325 \pm 36	1.00	1139 \pm 34	1.00
aphidicolin	194 \pm 31	0.60	1071 \pm 9	0.94
ddTTP	322 \pm 33	0.99	875 \pm 19	0.77
both	88 \pm 17	0.27	467 \pm 52	0.41

^aDamage-specific fmoles dNMP incorporated per 300 ng DNA. Values given are the mean and standard deviation of two experiments.

Incorporation of radiolabeled dCMP into methylene blue-damaged DNA by extracts from normal and repair deficient cells

To examine the effects of defective NER on the repair of this oxidatively damaged DNA, we determined the *in vitro* DNA repair synthesis performed by extracts from apparently normal donors, *xeroderma pigmentosum* (XP) and Cockayne syndrome patients. The results are shown in Figure 6. The image of the ethidium bromide-stained gel shows even loading of the damaged and undamaged plasmids and the autoradiogram shows specific incorporation of radiolabeled dCMP into the methylene blue-damaged plasmid. The damage specific incorporation of radiolabeled dCMP by the two normal cell lines, GM01310 and AG09387, were within 15% of each other. The XPA and XPB cell lines showed considerably less repair activity toward the methylene blue-damaged DNA than the normal extracts (25–40%), but the XPD line, GM02253e, was about twice as active as the normal extracts. Experiments using 50 μ g each of XPA and XPB WCE result in a partial complementation of damage-specific dCMP incorporation (data not shown). The CSA line, GM01857, exhibited 140% of the incorporation of radioactive dCMP compared with the normal but the CSB line, GM01712, was somewhat deficient in this repair, exhibiting 67% of the activity compared to the normal.

The observation that cell extracts from two different XP complementation groups exhibit a deficiency in repair suggests that some XP proteins may directly or indirectly influence the repair of 8-oxodG detected by the incorporation of dCMP. However, the proficiency of XPD extracts in this repair suggests that it is not NER *per se*. CS extracts, shown here to be proficient or nearly proficient in the repair of methylene blue-induced damage, are also proficient in the *in vitro* repair of UV-induced damage (29,34).

Models of DNA repair pathways

The considerable specificity of damage produced by methylene blue permits differences in dGMP and dCMP incorporation to be interpreted in terms of the size of the repair patch. Based on the average ratio of dGMP to dCMP incorporation of $r_{\text{obs}} = 2.7 \pm 0.5$, the average repair patch size of $n = 3.3 \pm 0.7$ is calculated by interpolation using equation 2. The use of a model assuming a random sequence in the repair patch 3' to the modified G, with an equal proportion of each nucleotide, does not produce a significantly different result. This implies that the *in vitro* repair of the oxidative damage lesion, 8-oxodG, when present in plasmid DNA, does not simply occur by the single nucleotide replacement pathway. Either the repair is dominated by a pathway that replaces approximately two adjacent nucleotides in addition to the modified guanosine, or two pathways contribute significantly to the repair. An average repair patch size of ~ 4 was determined for the repair of a synthetic (reduced) abasic site in *Xenopus laevis* oocyte extracts (35).

Differences in the kinetics of incorporation of dCMP compared to dGMP (Fig. 5) and differences in the effects of inhibitors (Table 1) suggest the involvement of at least two pathways in the repair of 8-oxodeoxyguanine. If we assume that the two pathways are single nucleotide replacement and 'short patch' repair with an average patch size of $m = 6$, then the ratio of dCMP and dGMP incorporated can be used to determine the relative fraction of the

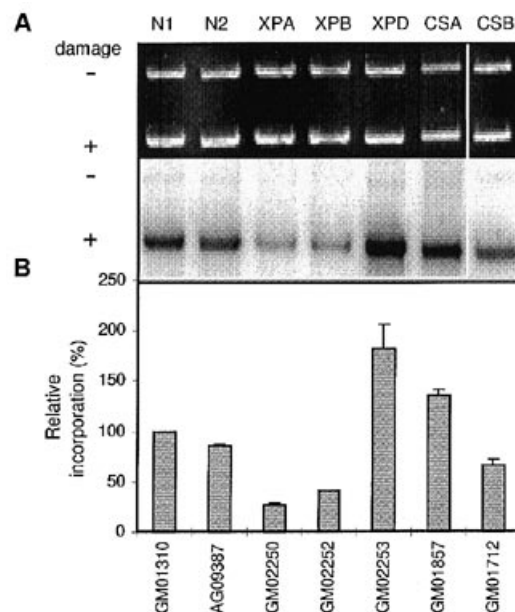


Figure 6. Damage-specific incorporation of [α - 32 P]dCMP by WCE from normal and repair defective cells into DNA damaged by methylene blue and visible light. (A) Gel images showing ethidium bromide staining of DNA and incorporation of [α - 32 P]dCMP. (B) Histogram showing damage-specific incorporation into methylene-blue damaged DNA. Data represent the mean \pm standard deviation of two experiments and are normalized to GM01310b.

activity proceeding by each path, using equation 3. The fraction of lesions repaired by the 'short patch' pathway is then calculated as $f_b = 0.56 \pm 0.15$ indicating approximately equal distribution of repair through the two pathways. Note that the choice of larger values for the size of the patch results in lower estimates of the contribution of the 'short patch' pathway. On the other hand, if the two pathways involved are single nucleotide replacement and nucleotide excision repair, with an average repair patch size of $m = 28$, then the corresponding fraction processed by the second pathway is $f_b = 0.11$, assuming that the repair patch has a random sequence composition. In this case, most of the lesions are processed by the single nucleotide replacement pathway and the nucleotide excision repair pathway only contributes modestly.

DISCUSSION

We have inferred characteristics of the repair of 8-oxodG in human cells by examining the damage-specific nicking and DNA repair synthesis activity of whole cell extracts on covalently closed circular DNA containing damage induced by methylene blue and visible light. Most of the damage is repaired by a BER process rather than NER as indicated by several observations, including the overall efficiency of repair of methylene blue-induced damage compared to UV-induced damage, the identification of DNA synthesis rather than incision as rate-limiting and the proficiency in the repair of this oxidative damage by XPD extracts, which are defective in the repair of UV-induced damage. These observations do not preclude a small contribution from NER (21). The involvement of more than one pathway in this efficient repair is suggested by the differences in the kinetics of dGMP and dCMP incorporation and differences in the sensitivity of incorporation of the two nucleotides to the inhibitors aphidicolin and ddTTP. Deficient repair of 8-oxodG

lesions by XPA and XPB extracts, as detected by incorporation of dCMP, suggests that some XP proteins may directly or indirectly influence the repair of this lesion.

Many studies indicate that 8-oxodG is repaired rapidly in human cells. Fpg protein-sensitive sites produced by photoactivated acridine orange, which include 8-oxodeoxyguanosine and formamido-pyrimidine, are rapidly repaired in both the nucleus and mitochondria, with >80% of the damage removed from actively transcribed DNA within 2 h, based on the gene-specific repair assay (36). An examination of the repair of specific base lesions induced by H₂O₂ in human cells, using the GC/MS technique, has shown that 8-oxoguanosine and 8-OH-adenine were removed more slowly than pyrimidine lesions and some other purine lesions (37). About 50% of the 8-oxodG is removed in 1 h (37). The rapid repair of the oxidative damage can be compared with the considerably slower repair of cyclobutane dimers in actively transcribed genes, which requires 24 h to repair 80% of the lesions (38), suggesting that most 8-oxodG lesions are removed by base excision repair processes.

However, some evidence implicates the mammalian nucleotide excision repair pathway in the repair of 8-oxodG. A shuttle vector containing a single 8-oxodG was defective in a plasmid replication assay after passage through XPA cells compared with normal cells, suggesting that XPA cells are defective in the repair of 8-oxodG (39). However, no significant impairment in the repair of methylene blue plus light induced damage was detected in XPA cells using a host cell reactivation assay (40). Plasmid DNA damaged by γ -irradiation or H₂O₂ and Cu²⁺ treatments, which produce a variety of base damages, did not show any significant difference in DNA repair synthesis between normal and XP cell extracts (41). However, after treatment of the plasmids with the *E. coli* enzymes Fpg protein and endonuclease III, the remaining damage was repaired in normal but not XP extracts (41). Recently, the excision of the characteristic 8-oxodG-containing oligonucleotide by the NER pathway using cell extracts or purified repair proteins has been demonstrated (21). The overall efficiency of that process is low and does not account for the efficient repair we observe here. Based on the 2-fold greater efficiency of excision of 8-oxodeoxyguanosine by the NER system compared with a cyclobutane dimer (21), we estimate that only a small fraction of the incorporated dCMP can be attributed to nucleotide excision repair.

Although cells from XP patients are considered to have normal activity in BER, some reports suggest a compromised ability to repair oxidative damage. Here we have shown that cell extracts from the XPA lymphoblastoid cell line, GM02250, exhibited considerably less [α -³²P]dCMP incorporation into DNA containing methylene blue induced damage compared with normal cell extracts. As discussed above, extracts from this cell line are also defective in the repair of Fpg protein- and endonuclease III-resistant lesions produced by γ -irradiation or treatment with H₂O₂ and Cu²⁺ (41). Fibroblast cells derived from the same patient (XP12BE) exhibited ~80% of normal repair of γ -irradiation induced damage in MT genes, based on the incorporation of BrdU, and were slightly sensitive to γ -irradiation (42). However, repair of thymine glycols in the same fibroblast cells is normal (43).

Although CS has been characterized as being defective in transcription-coupled NER, various CS lines also exhibit radio-sensitivity (42). CS3BE fibroblasts derived from the same patient as the CSA line studied here exhibits only a slight increase in

sensitivity (42), while a different CSB patient (CS1AN) was quite sensitive. The radiosensitivity of CS cells has recently been attributed to the requirement for CSB protein in the transcription-coupled repair of thymine glycols, another oxidative damage lesion in DNA also thought to be repaired predominantly by BER (43). The global repair of thymine glycols was not defective in CSB cells (43), which is similar to the absence of a significant defect in the *in vitro* repair of 8-oxodG lesions shown here.

BER of uracil from DNA in mammalian cells proceeds predominantly by a single nucleotide replacement mechanism involving DNA polymerase β (32). Recently, the existence of an alternative, PCNA-dependent pathway has been demonstrated for the resolution of repair initiated by glycosylases in *Xenopus* (44), hamster (45) and human extracts (46). The dependence of this second pathway on PCNA may arise either through the involvement of DNA polymerase δ or ϵ (33,47), in which PCNA loads polymerase δ or ϵ onto the DNA, or through PCNA stimulation of FEN-1 activity (46). Although this second pathway has been demonstrated to operate on reduced or oxidized AP sites (46), it may also be an essential feature of the repair of oxidized base damage recognized by eukaryotic glycosylases that proceed by a mechanism similar to *E. coli* endonuclease III, creating a gap bounded by a DNA strand with a 3' unsaturated aldehyde (14).

In this paper, we have presented evidence that a common oxidative lesion in DNA is efficiently repaired by at least two pathways in human cells. These pathways may be initiated by a glycosylase specific for the removal of 8-oxodG such as the hOGG1 protein. Following the initial nicking, the repair may proceed through single base replacement or by a slower process that involves the replacement of the 8-oxodG and several surrounding bases. These repair pathways may be similar to the PCNA-dependent and independent pathways which have been previously described.

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