Oxidative Stress–Induced Protein Damage Inhibits DNA Repair and Determines Mutation Risk and Therapeutic Efficacy

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Abstract

The relationship between sun exposure and nonmelanoma skin cancer risk is well established. Solar UV (wavelength 280–400 nm) is firmly implicated in skin cancer development. Nucleotide excision repair (NER) protects against cancer by removing potentially mutagenic DNA lesions induced by UVB (280–320 nm). How the 20-fold more abundant UVA (320–400 nm) component of solar UV radiation increases skin cancer risk is not understood. Here it is demonstrated that the contribution of UVA to the effect of UV radiation on cultured human cells is largely independent of its ability to damage DNA. Instead, the effects of UVA reflect the induction of oxidative stress that causes extensive protein oxidation. Because NER proteins are among those damaged, UVA irradiation inhibits NER and increases the susceptibility of the cells to mutation by UVB. NER inhibition is a common consequence of oxidative stress. Exposure to chemical oxidants, treatment with drugs that deplete cellular antioxidants, and interventions that interfere with glucose metabolism to disrupt the supply of cellular reducing power all inhibit NER. Tumor cells are often in a condition of oxidative stress and one effect of the NER inhibition that results from stress-induced protein oxidation is an increased sensitivity to the anticancer drug cisplatin.

Implications: As NER is both a defense against cancer and a significant determinant of cell survival after treatment with anticancer drugs, its attenuation by protein damage under conditions of oxidative stress has implications for both cancer risk and for the effectiveness of anticancer therapy. Mol Cancer Res; 14(7); 612–22. ©2016 AACR.

Introduction

The UV in sunlight causes skin cancer. Direct absorbance of UVB (280–320 nm) by DNA produces the cyclobutane pyrimidine dimers (CPD) and pyrimidine 6:4 pyrimidone adducts (6:4 Py:Ps) that are the major DNA lesions induced by solar radiation (1). These lesions are responsible for the signature C to T changes at PyC sequences that dominate skin cancer mutation spectra (reviewed in ref. 2). The nucleotide excision repair (NER) system removes UVB-induced DNA photoproducts. This prevents mutation and protects against skin cancer. The impaired NER, extreme photosensitivity, and skin cancer susceptibility in individuals with the genetic disorder xeroderma pigmentosum established the paradigm for the inverse relationship between effective DNA repair and cancer risk (3). Despite the protection afforded by NER, skin tumors (4–7) and even morphologically normal skin (8) accumulate extremely high numbers of UVB signature mutations. Surprisingly, mutation frequencies in nonmelanoma skin cancers (NMSC) in particular approach those in tumors in which disabled replication error-correcting systems confer extremely high spontaneous mutation rates (9). There are, however, no known deficiencies in DNA repair or editing in NMSCs or normal skin and it seems paradoxical that such large numbers of UVB-induced mutations coexist with an NER system dedicated to removing mutagenic DNA photolesions.

The contribution to cancer risk of UVA (wavelength 320–400 nm) that comprises >95% of incident solar UV radiation is enigmatic. Although UVA is classified as a human carcinogen by the WHO (10), it causes little direct DNA damage. UVA is, however, a source of reactive oxygen species (ROS) via interactions with cellular chromophores. DNA damage caused by UVA-induced ROS (11) is a potential contributor to sun-induced mutation and cancer. The signature mutations of oxidative DNA lesions are not, however, prominent in the mutational landscapes of skin tumors (12).

We have previously shown that some of the effects of UVA on cultured cells and skin are mimicked and amplified when UVA is combined with an exogenous UVA chromophore. Among these, the purine analogue 6-thioguanine (a metabolite of the immunosuppressant azathioprine) and fluoroquinolone antibiotics are mixed Type I and Type II UVA photosensitizers that induce oxidative stress, an excess of ROS. The ROS generated in these photosensitized reactions cause widespread DNA damage. They also induce extensive protein oxidation (13, 14). One important consequence of this extensive oxidation is the inactivation of DNA repair by damage to essential proteins. These include the RPA single-strand DNA-binding complex that is essential for NER (15) and cells treated with UVA/photosensitizer combinations have a reduced capacity to excise canonical UV photoproducts.
The extreme DNA damage resistance of certain bacteria (16) and microscopic aquatic animals (17) emphasizes the importance of preventing protein oxidation. These organisms deploy sophisticated antioxidant systems to maintain the integrity of cellular survival systems and protect them against the extreme oxidative stress that accompanies desiccation. The prevention of protein damage and maintenance of functional DNA repair is a major contributor to their enhanced resistance (18).

Several antioxidant systems protect human cells against oxidative stress. These forestall the potentially dangerous consequences of protein oxidation such as the formation of oxidized protein aggregates. Treatment with oxidants or changes in metabolism can perturb the balance between the formation and removal of ROS and induce oxidative stress. The altered metabolism of cancer cells means that many exist in conditions of sustained oxidative stress, a characteristic that is particularly associated with oncogenic transformation (19). Intracellular ROS levels can be an important determinant of the effectiveness of anticancer drugs. Pharmacologic interventions to decrease antioxidant protection are generally associated with enhanced sensitivity to anticancer agents. It is noteworthy that many of these kill tumor cells by inflicting potentially lethal DNA lesions that are substrates for DNA repair.

Although the contribution of oxidative DNA damage to cancer risk has been widely studied, relatively little attention has been paid to the possible influence of protein oxidation. The vulnerability of the DNA repair proteome to inactivation by oxidation by photosensitized UVA raises the possibility that DNA repair might be compromised under conditions of oxidative stress. In particular, that NER in skin cells might be partially inactivated by oxidative stress induced by solar UVA. In this report, we describe the impact of oxidative stress on DNA repair efficiency in immortalized HaCaT human keratinocytes and other cultured human cells. In particular, we show that DNA repair, including NER, is inhibited by treatment with UVA radiation or oxidizing chemicals. Interventions that alter glucose metabolism and deplete antioxidant defenses also inhibit NER providing further evidence for the involvement of oxidative stress. The vulnerability of DNA repair to inactivation by protein oxidation has implications for mechanisms of carcinogenesis and for the effectiveness of anticancer therapies.

Materials and Methods

Cell culture and treatments

All cell lines were obtained from Clare Hall Laboratory Cell Services. HaCaT (immortalized human keratinocyte) HeLa, HT1080, U2OS, and U2OS-RPA21 (15) cells were cultured in DMEM, TK6 in RPMI. Media were supplemented with 10% FCS. All cell lines had been authenticated by short tandem repeat profiling within 12 months prior to use. Tests for mycoplasma contamination performed at the same time were negative. Buthionine-sulfoximine (BSO) treatment was 2 mmol/L for 24 hours prior to irradiation. D,L-D2O treatment for 30 minutes. For D2O treatment, cells were held for 30 minutes prior to irradiation in PBS/D2O. Vitamin C treatments were for 3 hours prior to irradiation and cells were cultured in vitamin C-containing medium during subsequent repair incubations. The mutant IDH1 inhibitor AG5198 was obtained from Millipore. To assay cell survival, treated cells were seeded into 6-well plates at a density of 300 cells/well and colonies stained and counted 10 days later.

UV irradiation

Cells were irradiated in PBS. UVA was delivered using a UVH 250 W iron bulb (UV Light Technology Limited, emission maximum 365 nm) at a dose rate of 0.1 kJ/m2/s. UVB radiation (maximum 312 nm) was from a LF-215 60 W bulb (Uvitec Limited) at 5 J/m2/s. UVC (254 nm) was delivered by a Stratalinker UV Crosslinker (Stratagene) at 10 J/m2/s.

ROS measurement

Cells were incubated in PBS containing CM-H2DCFDA (Life Technologies) for 20 minutes at 37°C, irradiated, and analyzed by FACs.

Oxidized protein detection

Protein carbonyls. Cell extracts in RIPA buffer were incubated with 50 µg/mL Alexa Fluor 647 Hydroxyamine (FHA; Invitrogen) for 2 hours at 37°C. Treated proteins (20 µg) were separated by 10% or 12% SDS-PAGE gels. Increased protein carbonyl levels were inferred from visual inspection of the fluorescent gels. As a control for protein loading, identical gels run in parallel were stained with Coomassie blue.

Protein sulfenates. Cells were lysed in RIP buffer containing 1 mmol/L biotin-1,3-cyclopentanedione (BP-1) probe (KeraFAST) and free thiols blocked by the immediate addition of 20 volumes 0.1% SDS) M280 Streptavidin Dynabeads (Invitrogen). After rotation overnight, the gel was washed sequentially with 2 mol/L urea, 1 mol/L NaCl, 0.1% SDS/10 mol/L DTT, and PBS (30 minutes each). Derivatized proteins were recovered by boiling and analyzed by PAGE and immunoblotting.

TK mutation assay

Mutagenicity was measured by induction of trifluorothymidine (F3Tdr)-resistant TK6 cells. Cultures were purged of preexisting mutants by 48-hour growth in HAT (100 µmol/L hypoxanthine, 0.4 µmol/L aminopterin, 16 µmol/L thymidine; Sigma) medium followed by 24 hours in HT (100 µmol/L hypoxanthine, 16 µmol/L thymidine). Twenty-four hours after return to normal medium, cells (0.5 × 10^5/mL in PBS) were irradiated, grown for 72 hours, and then seeded into 96-well plates at a density of 8,000 cells/well in selective medium containing 2 µg/mL F3Tdr. Plating efficiency was assessed by seeding 1.6 cells/well in normal medium. Positive wells containing surviving colonies were scored 10 days later. Mutation frequencies were calculated from the Poisson distribution and compared using the two-tailed Mann–Whitney test. Data are expressed as means ± SD of six independent determinations.

Photoproduc repair

Irradiated cells were returned to full medium at 37°C and sampled at different times. DNA was extracted using the QiAamp DNA Mini Kit (Qiagen) and 6:4 Py-Pys and CPDs measured by ELISA (Cosmo Bio). Data from all 6:4 Py-Py ELISAs of cells treated with either 200 J/m2 UBV or 200 kJ/m2 UVA + 200 J/m2 UBV were pooled to create historic values (9 and 6 replicates, respectively). Graphical data points represent the mean of at least two independent experiments ± SD.
DNA Repair Inhibition by Oxidative Stress

In vitro NER

In vivo NER

Dual-incision assays were performed according to the modified (13) method of Laine and colleagues (20).

Immunoblotting

Cell extracts (20 μg) in RIPA buffer were separated on either 3%–8% Tris-Acetate (MCM2) or 10% Bis-Tris gels (PCNA, RPA32, global protein sulfenates; Invitrogen), and transferred to Immobilon-P membranes (Millipore). Antibodies used were from Santa Cruz Biotechnology (MCM2, PCNA) and Abcam (RPA32, G6PD). Streptavidin was from Invitrogen. Binding was detected by ECL reagent (GE Healthcare).

NADPH measurements

NADPH was measured using the NADP/NADPH Assay kit (Abcam) according to the manufacturer’s instruction. Data are means ± SD of three determinations.

RNA interference

siRNAs against G6PD were On-target smart pools from Dharmacon and were transfected using RNAI Max (Invitrogen) according to the manufacturer’s protocol. Cells were analyzed 72 hours after transfection.

Results

UVA (but not UVB or UVC) generates ROS and inhibits DNA repair

UVA irradiation induces ROS in human cells. Figure 1A shows that exposure of HaCaT cells to 100 or 200 kJ/m² UVA generated ROS and caused a measurable increase in the levels of both protein carbonyls and cysteine sulfenates that are acknowledged markers of generalized oxidative protein damage (Fig. 1B and C and Supplementary Fig. S1). UVB and UVC were much less effective in generating ROS even at doses that induced approximately 10 times more canonical UV lesions as determined by ELISA. Consistent with lower levels of ROS induction, neither UVB nor UVC detectably increased protein carbonylation. The predominant DNA photoproduct of UVA radiation is the T°C-T CPD (11) but the formation of these photoproducts does not account for UVA cytotoxicity. At equivalent initial CPD levels, UVA is at least 5-fold more cytotoxic in HaCaT cells than UVB (Supplementary Fig. S2). This observation is consistent with earlier suggestions that the cytotoxicity of longer UV wavelengths is not simply related to the induction of CPDs in human fibroblasts (21).

As the initial photoproduct levels cannot account for UVA toxicity in HaCaT cells, we examined whether UVA affected the persistence of potentially lethal DNA lesions by interfering with NER. To analyze the efficiency of NER, we measured the rate of disappearance of 6-4 Py:Py photoproducts induced by UBV. These DNA-distorting lesions are excised promptly from human cells with a half-life of 2–4 hours. Consistent with this rapid repair, ELISA measurements indicated that four hours after irradiation with 200 J/m² UBV, HaCaT cells had excised approximately 70% of the initial 6-4 Py:Py photoproducts (Fig. 1D). When HaCaT cells were also exposed to UVA, which does not induce these lesions (11), the removal of 6-4 Py:Py photoproducts was impaired and the rate of their repair was reduced in a UVA dose–dependent manner. At 200 kJ/m² UVA, the rate of repair was less than 50% of that of cells that had not been exposed to UVA. Thus, NER is inhibited by UVA doses that induce detectable protein oxidation.

UVA irradiation also compromised the removal of CPDs, the more abundant photoproducts induced by UBV, UVB, and UVC all induce CPDs albeit with widely different efficiencies. To ensure an appropriate comparison, HaCaT cells were exposed to doses of UVC, UVB, and UVA that induced similar initial numbers of CPDs. Compared with 6-4 Py:Py, these photoproducts are repaired more slowly by NER and ELISA measurements indicated that 24 hours after irradiation, HaCaT cells had removed approximately 70% of the initial CPDs induced by 7 J/m² UVC or 300 J/m² UVB (Fig. 1E). This figure is consistent with the reported half-life of CPDs in NER-proficient human keratinocytes (22). In contrast, 24 hours after irradiation with 300 kJ/m² UVA, more than 50% of CPDs remained in HaCaT DNA (Fig. 1E). This observation is consistent with previous reports that human keratinocytes excise UVA-induced T°C-T CPDs more slowly than the same lesion induced by UVB (23, 24). Taken together, the extended persistence of UVA-induced 6-4 Py:Py and CPDs indicates that exposure to UVA compromises NER in HaCaT cells.

UVA also inhibited base excision repair (BER) of an oxidative DNA lesion. HaCaT cells were treated with KBrO₃ to induce DNA 8-oxoG, a substrate for removal by BER. ELISA measurements indicated that more than 60% of the initial DNA 8-oxoG was excised within 20–30 minutes. When KBrO₃ treatment was preceded by irradiation with 200 kJ/m² UVA,
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Figure 2.

UV-induced mutation. TK gene mutation frequencies in TK6 cells treated with UVB and UVA. F3TdrR mutations induced by 2 J/m² UVB, 20 kJ/m² UVA, and 50 kJ/m² UVA are presented along with mutation frequencies in cells treated with UVA/UVB combinations (2 J/m² UVB + 20 kJ/m² UVA, and 2 J/m² UVB + 50 kJ/m² UVA). "Additive" values are the aggregate frequency from cells treated with UVA or UVB separately. Comparisons are by a two-tailed Mann-Whitney test. *, P < 0.05; **, P < 0.01. A mean background frequency (3.8 ± 1.6) x 10⁻⁶ has been subtracted from each radiation-induced value.

HaCaT cells removed less than 25% of the initial DNA 8-oxoG in 30 minutes (Fig. 1F). The slower repair was not due to an increased DNA 8-oxoG level in irradiated cells. 8-oxoG was not detectable by ELISA following treatment with 200 kJ/m² UVA. This observation is in agreement with reports that UVA induces relatively few DNA 8-oxoG lesions (<0.1% of T<>T; ref. 11).

Oxidant treatment inhibits DNA repair

To examine whether NER inhibition was due to UVA-induced oxidative stress, we treated HaCaT cells with the oxidizing agent H₂O₂. Although H₂O₂ is unreactive, it is converted to ROS via the Fenton reaction and causes DNA and protein damage. H₂O₂ induced protein carbonylation (Fig. 1G) and inhibited the NER of UVA-induced DNA photoproducts by HaCaT keratinocytes. ELISA measurements indicated that four hours after UVB irradiation, H₂O₂-treated cells had only excised around 40% of 6-4 Py:Py excision by HaCaT keratinocytes. ELISA measurements indicated that four hours after UVB irradiation, H₂O₂-treated cells had only excised around 40% of 6-4 Py:Py excision by HaCaT keratinocytes. ELISA measurements indicated that four hours after UVB irradiation, H₂O₂-treated cells had only excised around 40% of 6-4 Py:Py excision by HaCaT keratinocytes.

UVA sensitizes cells to UVB mutagenesis

Because NER provides the main protection against mutation by solar UVB, we examined whether NER inhibition by UVB detectably affected UVA-induced mutagenesis. Figure 2 shows that UVA and UVB are synergistically mutagenic in the standard TK6 mutation assay (25). Initial measurements determined that 2 J/m² UVB approximately doubled the background frequency of (3.8 ± 1.6) x 10⁻⁶ trifluorothymidine resistant (F3TdrR) TK6 mutants. Cells were then irradiated with 2 J/m² UVB combined with 20 or 50 kJ/m² UVA and the mutant frequency was compared with the sum of the frequencies induced by the same doses of UVB or UVA alone. A combination of 2 J/m² UVB with 20 kJ/m² UVA induced (25.0 ± 11.8) x 10⁻⁶ F3TdrR mutants. This value is more than 2-fold (P = 0.041) higher than (10.6 ± 14.1) x 10⁻⁶ which is the sum of the frequencies (3.6 x 10⁻⁶ + 7.0 x 10⁻⁶) induced by 2 J/m² UVB and 20 kJ/m² UVA, respectively. When cells were irradiated with a combination of 2 J/m² UVB and 50 kJ/m² UVA, the F3TdrR frequency increased to (53.7 ± 29.2) x 10⁻⁶. This value is again significantly higher than (19.1 ± 7.4) x 10⁻⁶ which is the aggregate frequency for 2 J/m² UVB and 50 kJ/m² UVA (3.6 x 10⁻⁶ + 15.5 x 10⁻⁶).

These findings indicate that the risk of mutation by combined UVA and UVB is higher than from the same doses delivered separately. They are consistent with UVA-mediated attenuation of NER and the persistence of potentially mutagenic DNA photoproducts.

Protein damage is responsible for DNA repair inhibition

To assess directly the possible involvement of protein damage in UVA-induced inhibition of DNA repair, we determined the ability of extracts from irradiated cells to perform NER in vitro. These experiments were carried out using HeLa cells. Preliminary measurements by ELISA confirmed that UVB inhibited 6-4 Py:Py excision by HeLa cells in vitro and that the extent of inhibition was comparable with that in HaCaT cells (Supplementary Fig. S3).

Extracts prepared from UVA-irradiated HeLa cells were less proficient in excising a damage-containing oligonucleotide from a platinated plasmid in a standard NER assay (ref. 13; Fig. 3A, top) and this effect was UVA dose-dependent. Neither UVB nor UVC affected in vitro NER. Excision by extracts from cells that had been irradiated with UVB and UVC (200 J/m²; 20 J/m²) to induce high levels of DNA photoproducts but no measurable protein oxidation, was comparable with that by extracts from unirradiated cells (Fig. 3A, bottom). The values from three independent assays are shown in Fig. 3B. Quantitation indicated that 200 kJ/m² UVA inhibited NER by more than 50%. The impaired NER activity in extracts of UVA-treated cells provides unequivocal evidence that protein damage underlies the attenuation of NER by UVA.

The RPA DNA-binding complex is particularly susceptible to inactivation by oxidation in vitro (26). We previously showed by complementation analysis with purified NER factors that damage to RPA is responsible for NER inhibition in cells exposed the oxidizing conditions induced by UVB activation of DNA 6-thioguanine (13). The observation that RPA overexpression in the U2OS-RPA21 cell line protects against NER inhibition further implicates oxidation damage to RPA in the effects of photosensitized UVA (15). Comparison by ELISA of NER by UVA-treated U2OS-RPA21 and the parental U2OS cells confirmed that RPA overexpression also confers protection against NER inhibition by UVA (Supplementary Fig. S4). As RPA thiol groups are particularly susceptible to inactivating oxidation (26), we probed extracts of UVA-irradiated HeLa cells for oxidized RPA thiols. Figure 3C shows that this form of oxidation of the NER-limiting RPA complex was significantly increased in HeLa cells that had received doses of UVA that inhibited NER. Overall the data suggest that oxidation damage to RPA underlies NER inhibition by UVA.
Proteins are particularly susceptible to oxidation by singlet oxygen (\(^{1}O_2\); ref. 27). \(^{1}O_2\) is a significant product in UVA-irradiated cells. The longevity and reactivity of \(^{1}O_2\) is increased in D\(_2\)O. Consistent with an enhanced reactivity with cellular proteins, protein carbonyl levels were higher when HaCaT cells were UVA irradiated in D\(_2\)O (Fig. 3D). The increased protein oxidation was accompanied by significantly enhanced NER inhibition and irradiation in D\(_2\)O with 200 kJ/m\(^2\) UVA effectively abolished 6:4 Py:Py excision by HaCaT cells (Fig. 3E). These observations provide further evidence for the involvement of protein oxidation in NER inhibition and indicate that \(^{1}O_2\) contributes to NER impairment by UVA in HaCaT cells.

Enhanced oxidative stress increases UVA-mediated DNA repair inhibition

The relationship between oxidative stress and NER inhibition was probed further by directly depleting antioxidant defenses to increase steady-state ROS levels. The antioxidant glutathione (GSH) is essential for redox homeostasis. As expected, inhibition of GSH synthesis by treatment of HaCaT cells with the glutamylcysteine synthetase inhibitor BSO decreased the levels of cellular GSH by at least 99%. The oxidative stress associated with BSO treatment elevated the steady-state protein carbonylation level and this was further increased by UVA irradiation (Fig. 4A). Immunoblotting confirmed the increased protein damage and revealed that BSO treatment increased intersubunit crosslinking in the PCNA and MCM protein complexes, additional markers of oxidative protein damage (Fig. 4B). Measurements of 6:4 Py:Py excision by ELISA. D\(_2\)O data represent the mean of two independent experiments.
with a sensitization of ovarian carcinoma cells to killing by carboplatin in culture and in xenografts (29). Treatment of HaCaT cells with high ascorbate concentrations induced oxidative stress and depleted NADPH levels (Fig. 4D). NADPH is essential for the regeneration of reduced GSH and the maintenance of redox homeostasis. Ascorbate treatment of HaCaT cells induced a dose-dependent increase in protein carbonylation and attenuated the excision of UVB-induced 6:4 Py:Pys by NER (Fig. 4E and F).

Figure 4. BSO and ascorbate-induced oxidative stress and NER. A, protein carbonyls in HaCaT cells treated with BSO (2 mmol/L, 24 hours) and 200 kJ/m² UVA. B, covalent PCNA and MCM2 crosslinking. Extracts from HaCaT cells treated with BSO (2 mmol/L, 24 hours) and UVA as indicated were analyzed by immunoblotting. Crosslinked PCNA (PCNA*) and crosslinked MCM2 species are indicated. C, NER by BSO-treated cells. ELISA measurements of 6:4 Py:Pys induced by 200 J/m² UVB in HaCaT cells pretreated with 200 kJ/m² UVA, or BSO + 200 kJ/m² UVA. Data for BSO represent the mean of two independent experiments. D and E, HaCaT cells were incubated in the presence of ascorbate (Vit C) at indicated doses for 3 hours. NADPH levels (D) and protein carbonylation (E) were assayed immediately. F, cells were treated with 8 mmol/L ascorbate (Vit C) for 3 hours, irradiated with 150 kJ/m² UVA and then incubated in the presence of ascorbate for the indicated times. Removal of 6:4 photoproducts was measured by ELISA. Data are means of three experiments.
The inhibitory effects of ascorbate were additive with those of UVA and the combination of high ascorbate concentration with 150 kJ/m² UVA was sufficient to almost completely inhibit NER (Fig. 4F).

NADPH is essential for maintaining redox homeostasis and cells respond to increased oxidative stress by diverting glucose metabolism through the pentose phosphate pathway (PPP) that provides a major source of NADPH. Silencing of G6PD (Fig. 5A), the first and rate-limiting step of the PPP depleted HaCaT NADPH levels by approximately 50%. Treatment with UVA (200 kJ/m²) also depressed NADPH levels and the effect of irradiation was additive with that of G6PD silencing (Fig. 5B). G6PD silencing also enhanced protein carbonylation by UVA (Fig. 5C). It inhibited NER and potentiated UVA-induced NER inhibition. Four hours after irradiation with 200 kJ/m² UVA and UVB, G6PD silenced cells had excised less than 10% of the initial 6:4 Py:Pys (Fig. 5D).

To investigate further the connection between NADPH levels and NER inhibition, we used HT1080 chondrosarcoma cells. These cells are heterozygous for a R132H mutation in the IDH1 isoform of isocitrate dehydrogenase (iIDH) that catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate in the TCA cycle. This reaction also generates NADPH. The gain-of-function R132H mutation enables the reduction of α-ketoglutarate to α-hydroxyglutarate in a reaction that consumes NADPH and compromises antioxidant protection. The IDH1 inhibitor AGI5198 specifically targets the mutated enzyme and prevents NADPH depletion (32). As expected, treatment of HT1080 cells with AGI5198 boosted their NADPH level. Increased NADPH levels provided protection against the effect of UVA in HaCaT cells and AGI5198 treatment reversed both the NADPH depletion and the NER inhibition induced by UVA (Supplementary Fig. S5).

Cisplatin is a member of the platinum-based family of drugs that is particularly effective against testicular and ovarian carcinomas. The toxicity of cisplatin is largely dependent on the induction of DNA damage and it is well established that NER significantly attenuates the therapeutic effectiveness of platinum drugs by removing potentially toxic platinum–DNA lesions (33). Figure 5E shows that G6PD silencing sensitized HaCaT cells to killing by cisplatin. The enhanced cisplatin sensitivity in G6PD-silenced HaCaT cells is consistent with a significant reduction in NER activity by the oxidative stress induced by inhibiting the PPP.

Our experiments identify several interventions that cause inhibition of DNA repair. Although each of the treatments may have additional effects, they all share the ability to induce oxidative stress and to measurably increase protein oxidation. Taken together, our observations reveal a significant susceptibility of DNA repair to inhibition under oxidative stress conditions. Inhibition reflects damage to members of the DNA repair proteome. The impact on NER of quite modest UVA doses is additive with that of antioxidant depletion.

**Discussion**

DNA repair pathways remove potentially mutagenic and lethal DNA lesions produced by radiation or chemicals. In doing so, they provide protection not only against the initiation of carcinogenesis but also against the intended toxicity of many anticancer treatments. The sensitivity of DNA repair to inactivation by oxidative stress has significant implications for both cancer development and cancer treatment.
UVB radiation is a significant source of oxidative stress in human cells and both DNA and proteins are major targets for oxidation damage. UVB compromised the BER of the common DNA oxidation product, 8-oxoG, in HaCaT cells. The OGG-1 and MUTYH DNA glycosylases that are essential participants in the removal of DNA 8-oxoG by BER, are known to be susceptible to inhibition by oxidation (13, 34). The common S326C-OGG-1 variant that is particularly prone to oxidation (34) is associated with increased risk of several forms of cancer (35). The presence of an essential iron-sulfur cluster in MUTYH (36) may explain its oxidation sensitivity. We have previously shown that oxidative stress resulting from the interaction between UVA and photo-sensitizing drugs also inhibits the non-homologous end-joining (NHEJ) pathway of DNA double-strand break (DSB) repair (13).

In that case, oxidation damage to the essential dimeric KU DNA end-binding complex is responsible for repair inhibition. Damage to Ku also occurs during oxidative stress induced by G6PD silencing and this too is associated with NHEJ inhibition (37, 38). All of these observations support our conclusion that damage to DNA repair proteins and inhibition of DNA repair is a general consequence of oxidative stress. Somewhat surprisingly, the repair of DNA lesions induced directly by ROS (DNA 8-oxoG) or as a secondary consequence of ROS-induced DNA damage (DSBs) can itself be compromised by oxidation damage to DNA repair proteins. Although we acknowledge that other cellular systems are almost certainly damaged by UVA-induced ROS, the vulnerability of NER is particularly significant as it has implications for the mechanism by which solar radiation induces skin cancer.

The NER pathway, the major protection against skin cancer (1), can be abrogated in HaCaT cells by treatment with oxidizing chemicals, by UVA radiation and by treatments that deplete antioxidant defences. UVA is very poorly absorbed by DNA and induces little DNA damage. The predominant UVA photoproduct is the T=T CPD, whereas oxidized DNA bases are generated much less frequently (11). Despite the T=T CPD being the major photoproduct of both UVB and UVA, mutations at TT dinucleotides are not common in skin tumors. This presumably reflects error-free processing of this lesion by DNA polymerase photoproduct of both UVB and UVA, mutations at TT dinucleotides point to the existence of additional damage targets. Our findings demonstrate that the level of oxidative stress determines the effectiveness of DNA repair. The efficiency of NER was compromised by oxidative stress induced by chemical oxidants and UVA radiation as well as by depletion of cellular antioxidant levels. Sustained oxidative stress is a common property of cancer cells. Although attempts to exploit this by further increasing ROS levels to breach a toxic threshold have met with mixed success (47), recent evidence indicates that treatment with ascorbate may be successful in this regard. At high concentrations, ascorbate generates H2O2 that causes widespread oxidative damage via the Fenton reaction (28). Several studies have indicated that these pharmacologic ascorbate concentrations can potentiate the toxicity of ionizing radiation and anticancer drugs that induce DNA damage (29, 48). In our experiments, one consequence of exposure of HaCaT cells to high ascorbate concentrations was NER inhibition by protein oxidation. The effect of ascorbate on NER was additive with that of UVA. Supplementing conventional carboplatin-based chemotherapy regimens with ascorbate increases DNA damage levels and toxicity in cultured ovarian tumor cells and improves the response to carboplatin-based therapy in ovarian tumor xenografts and in ovarian cancer patients (29). Carboplatin and the closely related cisplatin are members of the platinum family of DNA-damaging anticancer drugs. A significant part of their toxicity is due to the induction of potentially lethal intrastrand DNA crosslinks. These lesions are good substrates for removal by NER, and NER status is an important determinant of the effectiveness of platinum-based therapy. The intrinsically low NER capacity of testicular carcinoma cells (49) is a significant contributor to the spectacular success of platinum therapy against these tumors. In our experiments, high concentrations of ascorbate inhibited NER in HaCaT cells and it seems likely that a part of the ascorbate-mediated improvement in the responsiveness of ovarian tumors to carboplatin-based therapy reflects a similar NER inhibition. Consistent with this possibility, we found that the increased oxidative stress that accompanies G6PD silencing also increased the sensitivity of HaCaT cells to cisplatin.

Conjugation to GSH is a common mechanism of drug detoxification that depletes antioxidant protection and can lead to oxidative stress. Cisplatin is one example of the many chemicals, including numerous anticancer drugs that both induce oxidative...
stress and inflict DNA damage. Additional protein oxidation–related NER inhibition by adjuvant treatment may further improve the therapeutic effect of these anticancer agents. The high burden of UVB signature mutations in NMSCs suggests that NER might be compromised by UVA. It is noteworthy in this regard that the signature mutations in many other tumors clearly reflect unrepairred DNA lesions (12) and NER attenuation by oxidative stress is a possible contributor to their development.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: E. McAdam, R. Brem
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. McAdam, R. Brem
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Oxidative Stress–Induced Protein Damage Inhibits DNA Repair and Determines Mutation Risk and Therapeutic Efficacy

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