Immune Effector Cells Produce Lethal DNA Damage in Cells Treated with a Thiopurine

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Abstract

Azathioprine, a widely used immunosuppressant, is also used in the control of inflammatory disorders. These are characterized by the local accumulation of immune effector cells that produce reactive oxygen species (ROS). The DNA of azathioprine-treated patients contains 6-thioguanine (6-TG), a base analogue that is particularly susceptible to oxidation. Here, we show that 6-TG is vulnerable to ROS produced by chemical oxidants and that cells containing DNA 6-TG are hypersensitive to these oxidants. We also show that 6-TG incorporated into the DNA of macrophages sensitizes them to killing by endogenously produced ROS. ROS generated by macrophages are also a hazard for cocultured nonmacrophage cells containing DNA 6-TG. This bystander vulnerability of cells containing DNA 6-TG to oxidation by ROS generated by immune effector cells has implications for the long-term use of azathioprine in the management of inflammatory disorders. [Cancer Res 2009;69(6):OF1–7]

Introduction

Chronic inflammatory disorders are relapsing and remitting conditions that reflect an underlying immune dysfunction. Chronic inflammation can be a risk factor for the development of malignancy, and patients with ulcerative colitis and Crohn's disease have an increased risk of colonic epithelial dysplasia and carcinoma (1). Current treatments for this group of diseases involve nonspecific suppression of the inflammatory processes with drugs, such as nonsteroidal anti-inflammatory drugs and steroids, antibiotics, and modulators of the immune response. In the latter category, the thiopurines azathioprine and 6-mercaptopurine (6-MP) are the treatments of choice for severe inflammatory bowel disease (IBD) and are often used in the management of rheumatoid arthritis (2). Thiopurines are efficacious and 70% of patients with steroid-dependent IBD achieve and maintain remission (3). Azathioprine is a prodrug that is cleaved to 6-MP, which in turn is metabolized to 6-thioguanine (6-TG) nucleotides (TGN). These are precursors of DNA synthesis and 6-TG becomes incorporated into DNA of patients taking azathioprine (4, 5).

DNA 6-TG has two properties that distinguish it from the canonical DNA bases. Firstly, it is a UVA chromophore with an absorbance maximum at 340 nm and is photochemically susceptible to UVA. One of the photoproducts of DNA 6-TG, guanine-6-sulfonate (G\(^{\text{SO}_3}\)), is a strong block to replication in vitro and in vivo. This lesion can, however, be bypassed by error-prone DNA polymerases (6) in an emergency response that is a potential source of mutation (7, 8). The corresponding photochemical reactions occur in patients taking azathioprine whose skin contains DNA 6-TG and is selectively sensitive to UVA wavelengths (9). The second important feature of DNA 6-TG is its low oxidation potential. The formation of G\(^{\text{SO}_3}\) reflects the relatively favorable oxidation of 6-TG by reactive oxygen species (ROS) generated photochemically by UVA.

Oxygen-related DNA damage is an inescapable hazard and cells have evolved multiple protective and repair systems to minimize the detrimental consequences of oxidized DNA, which include disease and aging. ROS are important signaling molecules and their intracellular levels are normally under careful control (10). Excessive production of ROS, following, for example, exposure to oxidizing chemicals such as H\(_2\)O\(_2\), causes oxidative stress and DNA damage. Persistent oxidative DNA damage is linked to human cancer (11). Chronic inflammatory disorders are characterized by the infiltration of a large numbers of neutrophils, monocytes, and lymphocytes into the affected sites (see ref. 12 for review). These immune effector cells are a significant source of ROS and they generate H\(_2\)O\(_2\) and superoxide (O\(_2^-\)) as part of their defense against pathogens. The persistence of inflammatory cells and the associated production of ROS are thought to play an important role in the pathogenesis of IBD. In addition, the duration and severity of inflammation are risk factors in the development of colon cancer in IBD patients (13, 14).

Systemic treatment with thiopurines introduces 6-TG into the DNA of all dividing cells, and we were interested to examine whether cells containing DNA 6-TG might be particularly at risk from ROS produced by immune effector cells. Here, we show that 6-TG is highly susceptible to oxidation by H\(_2\)O\(_2\), one of the main components of ROS generated by immune cells, and to KBrO\(_3\), another chemical oxidant, and that cells containing DNA 6-TG are hypersensitive to killing by these oxidants. To mimic the infiltration of ROS-generating cells into sites of chronic inflammation, target cells containing DNA 6-TG were cocultured with an established macrophage cell line. Our results show that ROS produced by macrophages oxidizes the DNA 6-TG of cocultured target cells and that this results in increased target cell death.

Materials and Methods

Unless stated, all chemicals were obtained from Sigma-Aldrich.

Cell culture. HCT116 human colon carcinoma, SV40-transformed GM0429f and MRC5-VA human fibroblast, and the murine macrophage precursor J774a.1 cell lines obtained from the Cancer Research UK, London Research Institute, Central Cell Services were grown in DMEM supplemented with 10% FCS (Life Technologies/Invitrogen). CCRF-CEM leukemia cells were grown in suspension culture in RPMI 1640 supplemented with 10% FCS. HCT116, GM0429f, and MRC5-VA cells were maintained in 10% CO\(_2\), whereas the CCRF-CEM and J774a.1 cells were maintained at 5% CO\(_2\) in 37°C humidified incubators.
Thioguanine labeling and oxidant treatment. Cells were routinely cultured for 48 h in medium containing 0.5 or 1 μmol/L of 6-TG. 6-TG–treated cells were incubated further in growth medium containing a chemical oxidant: H₂O₂ (0.01–1 mmol/L, 1 h), KBrO₃ (1–100 mmol/L, 3 h), or ionizing radiation (IR; 1–10 Gy) delivered by a ¹³⁷Cs γ-radiation source in conjunction with an IBL 437C irradiator (2.2 Gy/min; CIS Bio International). UVC irradiation was delivered from a germicidal UVC lamp at a dose rate of 1.1 J/m²/s.

Clonogenic survival assay. For adherent cells, survival was determined by clonal assay in triplicate six-well plates. Surviving colonies were allowed to grow for 7 to 10 d. Giemsa stained, and counted. Each experiment was performed at least thrice. Results are expressed as mean ± SE.

Annexin and propidium iodide staining. In some experiments, two-color flow cytomtery was used to assess viability of HCT116 or CCRF-CEM cells. After 48-h coculture with J774a.1, all cells were harvested, washed twice with PBS, and incubated with Annexin V-FTTC (1 μg/ml) and propidium iodide (PI; 5 μg/ml) in binding buffer [10 mmol/L HEPES/NaCl (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L CaCl₂] for 15 min at room temperature in the dark. Samples were analyzed within 1 h of staining using a Becton Dickinson FACScan. J774a.1 cells were gated and size excluded. Quadrant markers were set on dot plots of unstained HCT116 (viable cell population) and then subsequently applied to other samples. Data acquisition and analysis was performed using CellQuest v3.3 software.

ROS detection. Cells were grown in the presence or absence of 6-TG (1 μmol/L) for 24 h, washed twice with PBS, and incubated with 5 μmol/L CM-H₂DCFDA (Invitrogen) for 20 min at 37°C. After additional washing with PBS, cells were treated with H₂O₂, KBrO₃, or IR. Treated cells were trypsinized and green fluorescence was analyzed by fluorescence-activated cell sorting (FACS).

Intracellular ROS production by lipopolysaccharide (LPS)–stimulated (10 μg/ml; Sigma-Aldrich) macrophages was examined by light microscopy. J774a.1 cells were incubated with 5 μmol/L CM-H₂DCFDA for 20 min at 37°C, washed with PBS, and examined for green fluorescence by microscopy [Zeiss Axioplan fitted with Hamamatsu C4747-95 digital camera images were acquired using OpenLab v2.2.5 (Improvement)].

ROS released by J774a.1 cells were quantitated by microassay (15). For H₂O₂, 100 μL of assay solution (28 mmol/L phenol red, 100 units/mL horseradish peroxidase in HBSS) were added to LPS-stimulated (1–10 μg/ml) J774a.1 cells in 96-well plates. After incubation for 1 h at 37°C, the reaction was stopped by the addition of 10 μL trisodium citrate (200 mmol/L) and 10 μL ethanol. RO₂⁻ was determined by absorbance at 505 nm (ε max, 324 nm; emission, ε max, 410 nm). This property, together with its elution time (Fig. 1B), identified it as the previously characterized G³⁴² deoxyguanosine (G³⁴²dR; ref. 7). Conversion of 6-TGdR to G³⁴²dR was dependent on H₂O₂ concentration between 0.01 and 1 mmol/L (Fig. 1C). We conclude that 6-TG is susceptible to chemical oxidation to G³⁴², a known product of photochemical oxidation.

DNA 6-TG sensitizes cells to chemical oxidants. HCT116 colorectal carcinoma cells were used to examine whether DNA 6-TG influenced sensitivity to the cytotoxic effects of chemical oxidants. These DNA mismatch repair-defective cells incorporate significant levels of DNA 6-TG without detectable toxicity. Following growth for 24 h in the presence of 1 μmol/L 6-TG, ~0.25% of the total DNA guanine was replaced by 6-TG. The 6-TG–treated cells were then exposed to H₂O₂, KBrO₃, or IR and their clonal survival was compared with that of similarly treated cells without DNA 6-TG. Figure 2A shows that DNA 6-TG caused a significant sensitization to H₂O₂. Formation of intracellular ROS

![Figure 1](image-url)
was confirmed using CM-H2DCFDA, a dye that emits green fluorescence on reaction with ROS (Fig. 2A, inset). 6-TG-treated HCT116 cells were also more sensitive to KBrO3 (Fig. 2B), which also generated measurable levels of intracellular ROS (Fig. 2B, inset). In contrast, the presence of DNA 6-TG did not significantly alter the sensitivity of cells to IR (Fig. 2C) under conditions that did not detectably increase steady-state levels of ROS (Fig. 2C, inset). We conclude that DNA 6-TG sensitizes cells to treatments that generate ROS. It is likely that the susceptibility of DNA 6-TG to oxidation underlies this effect.

The UVA photoproducts of DNA 6-TG, particularly DNA G\textsuperscript{SO3}, are robust inhibitors of DNA replication (8). In agreement with the likely formation of DNA G\textsuperscript{SO3}, H\textsubscript{2}O\textsubscript{2} induced a pronounced replication inhibition in HCT116 cells containing DNA 6-TG (Fig. 2D). These data are consistent with the oxidation of DNA 6-TG to replication-blocking DNA lesions, including G\textsuperscript{SO3}, and the enhanced sensitivity of 6-TG-treated cells to killing by oxidizing chemicals.

**Lethal oxidation products are not excised by nucleotide excision repair.** Nucleotide excision repair (NER) can excise potentially lethal lesions, which distort the DNA double helix (16). To investigate whether NER could ameliorate the lethal effects of oxidized DNA 6-TG, we compared the sensitivity of cell lines proficient or defective in NER, GM04429f cells, SV40-transformed fibroblasts derived from a xeroderma pigmentosum (XP) group A patient, and MRC5-VA, similarly transformed fibroblasts from a clinically normal individual, were cultured for 48 hours in 0.5 μmol/L 6-TG. This resulted in comparable levels of DNA substitution by 6-TG (0.22% DNA guanine in GM04429f and 0.26% in MRC5-VA). The presence of this relatively low level of DNA 6-TG did not detectably affect the viability of either cell line as determined by clonogenic assay (data not shown). Cells were treated with H\textsubscript{2}O\textsubscript{2} and clonal survival was measured. In agreement with published findings (17), GM04429f cells were ≈2-fold more sensitive than MRC5-VA to H\textsubscript{2}O\textsubscript{2} (Fig. 3). DNA substitution by 6-TG sensitized GM04429f cells to H\textsubscript{2}O\textsubscript{2} and induced a similar degree of sensitization in MRC5-VA cells. The intrinsic hypersensitivity to UVC of MRC0429f cells was confirmed in a parallel experiment in which cells not treated with 6-TG were exposed to UVC (Fig. 3, inset). Similar results were obtained with a second XPA cell line, XP12BRo, in which DNA 6-TG induced a similar degree of sensitization to H\textsubscript{2}O\textsubscript{2} (data not shown). These findings indicate that NER does not play a significant role in the removal of potentially cytotoxic DNA lesions produced by the chemical oxidation of DNA 6-TG.

**DNA 6-TG oxidation by ROS from immune effector cells.** As a model for ROS-secreting immune effector cells in an intense inflammatory microenvironment, we used the murine macrophage precursor cell line J774a.1. Microassays (15) confirmed that unstimulated J774a.1 cells secrete measurable H\textsubscript{2}O\textsubscript{2} and superoxide (O\textsubscript{2}\textsuperscript{-}) and that this is increased following treatment with LPS (Fig. 4A and B). ROS generation by J774a.1 cells was confirmed by fluorescent microscopy using CM-H2DCFDA dye. Within 1 hour of treatment with LPS, there was an ≈10-fold increase in the number of positively staining cells. Even after stimulation, however, only ≈10% of cells contained detectable ROS; a representative image is presented in Fig. 4C. The percentage of positive cells remained approximately constant for up to 48 hours after stimulation (Fig. 4D), although direct microscopic examination indicated that the secreting cell population changed with time, possibly indicating paracrine signaling. Overall, it was apparent that the high levels of ROS are maintained for at least 48 hours after LPS stimulation.

To examine whether DNA was vulnerable to oxidation by endogenous ROS, we compared DNA 6-TG oxidation in J774a.1 and HCT116 cells. Cells were cultured in the presence of 6-TG for 48 hours, DNA was extracted, and the extent of DNA substitution by

![Figure 2. DNA 6-TG sensitizes cells to killing by chemical oxidants that induce ROS. HCT116 cells that had been grown in the presence of 6-TG (1 μmol/L) DNA for 24 h were treated with (A) H\textsubscript{2}O\textsubscript{2} (1 h) or (B) KBrO3 (3 h) or (C) γ-irradiated at the doses indicated. A to C, survival of HCT116 in the absence of 6-TG (black line) or containing 6-TG in their DNA (gray line) was determined by clonogenic assay. Points, mean of four independent experiments; bars, SD. In parallel, the same HCT116 cells were loaded with the ROS-reactive dye CM-H2DCFDA before exposure to H\textsubscript{2}O\textsubscript{2}, KBrO3, or IR. A to C, inset, changes in fluorescence intensity were analyzed by FACS. The solid histogram represents untreated cells. D, DNA replication. Control (black line) or 6-TG-treated HCT116 cells (gray line) were treated with H\textsubscript{2}O\textsubscript{2} for 1 h and [\textsuperscript{3}H]TdR incorporation into TCA-insoluble material was measured. Incorporation is expressed as a percentage of that in untreated cells. Points, mean of two separate experiments; bars, SD.](image-url)
6-TG and the fraction of incorporated 6-TG that had been oxidized to G\(_{SO3}\) was determined by HPLC. In the ROS-producing J774a.1 cells, 0.4 ± 0.16% of incorporated 6-TG had been converted to DNA G\(_{SO3}\). This value is 5-fold higher than the 0.08 ± 0.05% DNA G\(_{SO3}\) associated with a similar level of 6-TG substitution in HCT116 cells. The increased ROS production that accompanied LPS treatment doubled the extent of DNA 6-TG oxidation in J774a.1 cells and G\(_{SO3}\) comprised 0.85% of incorporated 6-TG in stimulated cells (Fig. 5).

ROS produced by immune effector cells are released into the surrounding tissue. We examined whether DNA 6-TG in neighboring cells might also be susceptible to secreted ROS. To do this, we cocultured J774a.1 cells with CCRF-CEM leukemia cells, which grow in suspension and can be easily separated from the tenaciously adherent macrophages. CCRF-CEM cells treated for 48 hours with 6-TG to replace 2% to 2.5% of their DNA guanine were mixed with LPS-activated J774a.1 cells and cocultured for a further 48 hours. At the end of this period, nonadherent cells were recovered and their DNA was analyzed for 6-TG and G\(_{SO3}\). Figure 5B shows that in the absence of J774a.1 cells, 0.03% of the DNA 6-TG was present as G\(_{SO3}\). Following culture with activated J774a.1 cells, this value increased to 0.22%.

**Cytotoxicity of ROS from immune effector cells.** The accumulation of a high level of DNA G\(_{SO3}\) was associated with a loss of cell viability. Annexin V and PI staining and FACS analysis of J774a.1 cells that had incorporated 6-TG during 48-hour incubation revealed a 6-TG dose-dependent loss of viability over the subsequent 48-hour period (Fig. 6). LPS treatment to increase the amount of DNA G\(_{SO3}\) significantly enhanced the cytotoxicity (Fig. 5C). In contrast, the 5-fold lower level of DNA G\(_{SO3}\) in HCT116 was not associated with cell death and treatment of these cells with LPS did not affect cell viability. We conclude that DNA 6-TG in J774a.1 cells renders them vulnerable to their constitutively high levels of ROS, and in this model macrophage, spontaneous oxidation of DNA 6-TG contributes to the cytotoxicity of 6-TG treatment. This toxic hazard is exacerbated by the increased ROS production that follows LPS activation.

To investigate the cytotoxic effects of ROS generated by immune effector cells on adjacent cells in trans, 6-TG–treated HCT116 cells were cocultured with J774a.1 macrophages. HCT116 cells with or without DNA 6-TG were plated together with J774a.1. After coculture for 48 hours, all the cells were recovered and the viability of target HCT116 cells was assessed by FACS analysis with Annexin V and PI staining. Figure 6A shows a representative dot plot of 6-TG–treated HCT116 cells. Negative staining for Annexin V and PI confirms that the majority of these cells were viable. Coculture with J774a.1 reduced the viability of HCT116 cells as indicated by the increased fraction of PI-positive and/or Annexin V–positive cells (Fig. 6B). After coculture with J774a.1 cells, HCT116 cells were gated using forward and side scatter to exclude the larger and more granular J774a.1 cells from the analysis (Fig. 6B, inset). There was a small but reproducible reduction of ~20% in HCT116 viability in the presence of unstimulated J774a.1 cells and this was significantly augmented to >60% by prior activation with LPS. Figure 6C summarizes data from four independent experiments. Qualitatively similar results were obtained with 6-TG–treated CCRF-CEM cells as the target. LPS-activated J774a.1 cells reduced viability by 50% (Fig. 6C).
cell survival and the viability of cocultured HCT116 and CCRF-CEM cells was almost doubled by NAC treatment (Fig. 6C). These observations provide confirmation of the involvement of ROS in the oxidation of DNA 6-TG and the contribution of oxidized DNA 6-TG lesions to cell death.

We conclude that cells containing DNA 6-TG are particularly vulnerable to killing by ROS produced from activated immune effector cells. This hypersensitivity affects the effector cells themselves as well as closely adjacent cells. Reduced viability is correlated with the susceptibility of DNA 6-TG to oxidation.

Discussion

The cytotoxicity of 6-MP and its parent compound azathioprine is partly mediated by metabolism into TGNs (18, 19). Systemic 6-MP or azathioprine treatment causes 6-TG to accumulate in the DNA of proliferating cells and DNA 6-TG is detectable in patients’ lymphocytes (4, 5) and skin (7). 6-TG is more reactive than canonical DNA bases (20), and we are interested in the implications of this reactivity for the welfare of patients undergoing long-term thiopurine therapy. DNA 6-TG is cytotoxic via its interaction with DNA mismatch repair (reviewed in ref. 19). The effects of DNA 6-TG we report here were independent of mismatch repair status, however, and were similar in repair-deficient HCT116 and CCRF-CEM cells and repair-proficient MRC5-VA fibroblasts, two XPA fibroblast lines, and J774a.1 macrophages. Our findings reveal a new mechanism by which DNA 6-TG is hazardous to cells. Its susceptibility to ROS produced in a biological context has implications for its use as an anti-inflammatory agent.

Although DNA 6-TG levels in patients undergoing thiopurine therapy are lower than those in our cultured cells (7), they nevertheless have detectable consequences; the skin of azathioprine-treated patients is selectively hypersensitive to UVA (9). Systemic azathioprine or 6-MP treatment often continues for many months or years, entailing a concomitant risk of more subtle chronic effects. The acute UVA sensitivity of azathioprine patients’ skin reflects the relatively favorable oxidation of DNA 6-TG by photochemically generated ROS. Our present findings extend this observation and show a susceptibility to chemical oxidants H2O2 and KBrO3, both of which increase the steady-state levels of intracellular ROS. Cell death was predominantly by necrosis and both agents induced rapid replication arrest. All these observations are consistent with oxidation of cellular DNA 6-TG to GSO3, which is a powerful replication block (8).

The fate of oxidized DNA 6-TG in cells is not yet established, although our findings indicate that GSO3 is removed poorly from DNA, and these and other potentially harmful oxidative DNA lesions are likely to remain in the affected cells throughout their lifetime. Oxidation products of DNA 6-TG, including DNA GSO3, are essentially iatrogenic DNA lesions of recent origin, and it seems unlikely that specific DNA damage/repair responses have evolved to process them. GSO3 is bulky, causes significant destabilization of duplex DNA (8), and is therefore a potential substrate for excision by NER. DNA 6-TG produced a similar degree of sensitization to killing by ROS produced in a biological context, but the oxidative DNA adducts are not removed efficiently. This protective effect was accompanied by a significant increase in cell viability.

Protection by antioxidants. Antioxidants protected DNA 6-TG-containing target cells against oxidation of their DNA and against the cytotoxic effect of oxidation products. The formation of DNA GSO3 in CCRF-CEM cells cocultured with LPS-activated J774a.1 cells was largely (80%) prevented by pretreatment with N-acetylcysteine (NAC), an acknowledged ROS scavenger (Fig. 6D).

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inflammation and reduces the risk of malignancy. This protective effect may be partially offset by the consequences of accumulated irreparable DNA lesions in bystanders in the vicinity of ROS-producing cells. 6-MP is used in remission maintenance regimens

One important finding is that cells containing DNA 6-TG are also sensitive to biologically derived ROS of which immune effector cells are an acknowledged source. Sites of chronic inflammation are associated with sustained generation of ROS from the respiratory burst of infiltrating activated neutrophils and monocytes (21, 22). Macrophages are postmitotic in vitro. Their differentiation in the presence of 6-TG would, however, lead to its incorporation during the proliferative phase of their maturation. Our findings indicate that macrophages that contain DNA 6-TG are at risk from self-inflicted DNA 6-TG oxidation and their sustained high level of endogenous ROS swiftly leads to cell death. Our coculture experiments indicate a second potential threat of biological ROS: cells in the vicinity of activated macrophages are also at risk of DNA damage. These bystander cells also accumulated a significant burden of cytotoxic DNA GSO3 in a reaction that was prevented by free radical scavengers.

Azathioprine and 6-MP are effective immunomodulatory therapies for active IBD (23). Chronic inflammation is a predominant contributor to the development of epithelial cancers and IBD patients have an increased risk of bowel cancer (14, 24, 25). There is some evidence to suggest that azathioprine may reduce cancer risk (14), although other studies have revealed no significant effect (24–26). It is possible that the selective suicide of activated immune effector cells containing DNA 6-TG contributes to the reduction in inflammation and reduces the risk of malignancy. This protective effect may be partially offset by the consequences of accumulated irreparable DNA lesions in bystanders in the vicinity of ROS-producing cells. 6-MP is used in remission maintenance regimens for leukemia. We note that because rapidly dividing tumor cells will incorporate relatively high levels of DNA 6-TG, they may have enhanced susceptibility to killing by drugs that generate ROS. The possibility that 6-TG combination therapy with this type of agent might provide a synergistic anticancer benefit suggests a potential new therapeutic role for this long-established class of drugs.

In summary, cells containing DNA 6-TG are susceptible to killing by chemical oxidants. Replication-blocking, potentially lethal oxidized DNA 6-TG lesions are not excised by NER and accumulate in cellular DNA. In an in vitro macrophage cell model, sustained release of H2O2 and O2 is associated with rapid and fatal accumulation of DNA GSO3. DNA 6-TG–containing target cells cocultured with macrophages were also vulnerable to DNA oxidation and killing. In as far as this simple model mimics chronic inflammation of the gut during IBD and treatment with thiopurines, the findings suggest that the low oxidation potential of DNA 6-TG may influence the immunomodulatory effects of thiopurines and the possible long-term effects of such treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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