In vivo administration of O6-benzylguanine does not influence apoptosis or mutation frequency following DNA damage in the murine intestine, but does inhibit P450-dependent activation of dacarbazine

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Clinically relevant cancer chemotherapeutic alkylating agents such as temozolomide and dacarbazine induce apoptosis and are mutagenic via the formation of O6-alkylguanine adducts in DNA. The DNA repair protein O6-alkylguanine-DNA alkyltransferase (AGT) functions by dealkylating such adducts and can thus prevent apoptosis and mutagenesis. In attempts to maximize the clinical effectiveness of these alkylating agents, inhibitors of AGT such as O6-benzylguanine (BeG) have been developed. We show here that within murine small intestinal crypt cells, BeG administration does not alter the apoptotic response to the direct-acting methylating agents N-methyl-N-nitrosourea (MNU), temozolomide and N-methyl-N′-nitro-N-nitrosoguanidine. Furthermore, we show that BeG pretreatment fails to elevate the mutation frequency at the murine Dlb-1 locus following exposure to MNU. Consistent with these results, we show that intestinal AGT activity is effectively abolished by administration of 100 mg/kg temozolomide, even in the absence of BeG. In contrast, pretreatment with BeG transiently abolished the apoptotic response to the methylating prodrug dacarbazine. Activation of dacarbazine to its reactive intermediate has previously been shown to be cytochrome P450 dependent and we show here that pretreatment of mice with the cytochrome P450 inhibitor metyrapone also inhibits dacarbazine-induced apoptosis. Thus BeG increases neither the prevalence of apoptosis nor mutation frequency in the murine small intestine, but is capable of inhibiting P450-dependent prodrug activation. The positive implication from this study is that BeG treatment may not exacerbate the toxic and mutagenic effects of methylating agents within normal cells, although it may engender other adverse reactions through the suppression of cytochrome P450-dependent processes.

Introduction

The murine small intestine offers an ideal experimental system to study both apoptosis and mutation frequency in response to DNA damaging agents such as γ-irradiation and alkylating agents (1–3). The DNA repair protein O6-alkylguanine-DNA alkyltransferase (AGT) is expressed in the murine small intestine and is up-regulated in a p53-dependent manner in response to DNA strand breaks following ionizing irradiation (4). AGT functions by recognizing and removing specific alkyl lesions from DNA (5). The principal substrate is O6-methylguanine, which is the major toxic and premutagenic lesion induced by methylating agents. AGT-mediated repair occurs via transfer of the alkyl group to a cysteine residue in AGT (17). Dacarbazine is a DNA alkylating agent used in the treatment of metastatic melanoma and Hodgkin’s lymphoma (18). It is a prodrug requiring cytochrome P450-dependent N-demethylation to produce the active compound 5-(3-methyltriazen-1-yl)imidazol-4-carboxamide (MITC) (19,20), MITC is also generated when temozolomide reacts spontaneously with H2O (21). MITC decomposes to a methylating intermediate common to a variety of agents that result in the methylation of DNA with subsequent consequences for cell death and mutation.

One approach to directly determine the somatic in vivo mutation frequency relies upon detecting loss of function at the polymorphic genetic locus Dlb-1 (22). This locus determines the ability to bind the lectin from Dolichos biflorus in mouse intestinal epithelium. Mutation frequency can be scored by identifying clonal populations which are no longer capable of binding the lectin. We have previously shown that in vivo administration of BeG reversibly suppresses AGT function, but does not modulate the apoptotic response to temozolomide.

Abbreviations: AGT, O6-alkylguanine-DNA alkyltransferase; BeG, O6-benzylguanine; DBA, Dolichos biflorus agglutinin; dacarbazine, 5-(3,3-dimethyltriazen-1-yl)imidazole-4-carboxamide; MITC, 5-(3-methyltriazen-1-yl)imidazol-4-carboxamide; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; MNU, N-methyl-N-nitrosourea; PBS, phosphate-buffered saline.

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or another methylating agent, N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), in the small intestine. Furthermore, we have shown that BeG treatment did not enhance the apoptotic response in a mismatch repair-deficient background (13). We show here that AGT plays no role in modulating the apoptotic response or mutation rate in the normal epithelium of the murine intestine following exposure to the methylating agent N-methyl-N-nitrosurea (MNU) or the apoptotic response to cisplatin or γ-irradiation, but that it blocks dacarbazine-mediated cell death.

Materials and methods

Mouse colonies

All mice were maintained under non-barrier conditions and given a standard diet and water ad libitum.

AGT assay

Mice were killed and tissues of interest removed, snap frozen in liquid nitrogen and stored at −70°C until assayed as described previously (4). Briefly, tissue samples were defrosted and disrupted by sonication (10 s at 10 μm peak-to-peak, followed by 10 s at 16 μm peak-to-peak) in 1 ml of 50 mM Tris-Cl (pH 8.3), 1 mM EDTA, 3 mM diithothreitol containing 5 μg/ml leupeptin, after which 10 μl of phenylmethylsulphonyl fluoride (87 μg/ml in ethanol) was added. Insoluble cellular debris was removed by centrifugation for 10 min at 13,000 r.p.m. at 4°C. Aliquots of the supernatants were incubated for 2 h at 37°C with a calf thymus DNA substrate that had been methylated by reaction with [3H]MNU (14.5 Ci/mM; Amersham). Specific activities were calculated from the amount (fmol) of [3H]methyl groups transferred per unit amount of total protein in the extract under conditions where activity was proportional to the amount of total protein assayed. The protein concentrations of the extracts were determined using bovine serum albumin as a calibration standard.

Treatment protocol

Eight to 10 week old mice were given i.p. injections of temozolomide (100 mg/kg), cisplatin (10 mg/kg; David Bull Laboratories), dacarbazine (150 mg/kg; Sigma), MNNG (50 mg/kg) or MNU (100 mg/kg). BeG was administered at a dose of 60 mg/kg i.p. Metyrapone was used at a concentration of 100 mg/kg, after BeG administration mice were injected i.p. with a single dose of MNU. were observed between BeG- and BeG+ treated mice. One hour after BeG administration mice were injected i.p. with a single dose of MNU.

Apoptosis quantitation

At a specified time point following reagent injection or irradiation, a minimum of three animals were killed, the small intestine removed, flushed with water and fixed overnight in methocarn (4 parts methanol, 2 parts chloroform, 1 part acetic acid). Haematoxylin and eosin stained sections were made and apoptosis scored through the use of the Highly Optimised Microscope Environment (HOME) as previously described (23,24). A minimum of fifty half-crypts were scored per animal.

 Dilb-1 mutation assay

A cohort of 8–10 week old Dilb-1 h/a heterozygote mice were treated with BeG (60 mg/kg i.p.). A second identical cohort received no BeG. One hour after BeG administration mice were injected i.p. with a single dose of MNU. Twenty-one days later the mice were killed, their small intestines removed and flushed with water. Whole mount preparations of 15 cm of the intestine were stained with Doliocbas biflorus agglutinin (DBA)–peroxidase as described by Winton et al. (22). Villus roots not staining with the DBA–peroxidase conjugate were scored using a Wild stereo microscope and the results presented as number of mutations per 105 villi.

Results

BeG does not induce apoptosis within the murine small intestine

As previously shown, administration of 60 mg/kg BeG functionally depletes AGT activity in the small intestine for a period between 1 and 12 h post-drug administration (13). AGT inhibition may result in increased cell death as a consequence of failure to repair endogenous alkylated DNA damage. To test this hypothesis, apoptosis was scored over a time course following BeG administration. Cohorts of mice were injected with 60 mg/kg BeG at time 0. Apoptosis was scored at 0, 3, 6, 12, 24, 48 and 72 h after injection. No increase in the incidence of apoptosis was observed up to 72 h following BeG injection (data not shown). During this time period we also assessed the levels of necrosis within the sample histologically, as it remained possible that BeG was eliciting cell death through mechanisms other than apoptosis. We saw no increase in the levels of necrosis.

MNU-, cisplatin- and γ-irradiation-induced apoptosis in murine small intestine is unaffected by functional AGT depletion

We have previously shown that BeG administration did not modify enterocyte apoptosis following treatment with temozolomide or MNNG (13). We report here the effect of BeG administration on the apoptotic response to MNU, dacarbazine, cisplatin and γ-irradiation. These agents were chosen either to determine the effect of exposure to alkylating agents (MNU and dacarbazine) or to act as negative controls (cisplatin and γ-irradiation). Wild-type mice were pretreated with BeG (60 mg/kg) and 1 h later either 100 mg/kg MNU, 10 mg/kg cisplatin, 150 mg/kg dacarbazine or 4 Gy of γ-irradiation were administered. Comparative data on temozolomide- and MNNG-treated mice is reproduced here from Toft et al. (13). Apoptosis was scored in the small intestine 6 h later (Figure 1). No significant differences in the prevalence of apoptosis were observed between BeG- and non-BeG-treated animals following MNU, temozolomide, cisplatin or ionizing irradiation (P > 0.4 in all cases, Mann–Whitney U-test). However, dacarbazine-induced apoptosis was almost completely inhibited following BeG treatment (P < 0.01, Mann–Whitney U-test). Once again, we did not observe an increase in necrosis in any of the treatment protocols, indicating that these agents were not killing cells through mechanisms other than apoptosis, at least within the time frame studied.

The apoptotic response following low doses of temozolomide is not altered by BeG administration

To investigate the possibility that the doses of alkylating agents used in Figure 1 were depleting cellular pools of active AGT
and therefore rendering BeG treatment ineffective, very low doses of temozolomide (range 0.5–16 mg/kg) were administered to mice with and without BeG administration. Apoptosis was scored 6 h later (Figure 2). The rationale was that at low doses of temozolomide not all functional AGT activity would be abolished by the alkylating agent and thus, if AGT could actually alter the level of apoptosis, a difference between BeG-treated and non-treated mice may be revealed. No significant differences in the incidence of apoptosis were observed between BeG-treated and non-treated mice at any dose of temozolomide ($P > 0.07$ for all doses, Mann–Whitney $U$-test).

MNU-induced mutation frequency at the Dlb-1 locus is not influenced by BeG

Mutation rate within the murine small intestine following MNU was scored at the Dlb-1 locus using standard approaches (1, 22). The mutation frequency increased with increasing doses of MNU (Figure 3), but prior treatment with BeG did not result in a significant elevation in the mutation frequency at the Dlb-1 locus ($P > 0.05$ for both doses, Mann–Whitney $U$-test).

Temozolomide administration effectively ablates AGT activity in the intestine

One explanation for the failure to observe BeG-dependent differences in apoptosis and mutation frequency is that treatment with the methylating agent alone is sufficient to render AGT functionally inactive and that, further, BeG-mediated suppression is therefore largely irrelevant. To directly test this we exposed mice to varying doses of temozolomide and scored AGT activity in both the liver and intestine. As we have shown before, pretreatment with BeG markedly reduces resting levels of AGT activity at 6 h (13). In the liver this reduction is from $150 \pm 40.8$ to $13.3 \pm 21$ fmol/mg protein. In the intestine AGT activity falls from a lower resting level ($45.6 \pm 12$ fmol/mg) to become undetectable 6 h after BeG treatment (13). The effect of exposure to increasing doses of temozolomide was indeed to reduce levels of AGT activity, as shown in Figure 4. Following exposure to 100 mg/kg temozolomide, AGT activity was effectively depleted in both the liver and small intestine.

BeG inhibits the P450-dependent metabolic activation of dacarbazine

We have shown that exposure to BeG inhibits apoptosis induced by dacarbazine (Figure 1). In order to study the kinetics of this inhibition a single dose of BeG (60 mg/kg) was administered to wild-type mice at time 0. Mice were then subsequently injected with 150 mg/kg dacarbazine at 0, 1, 6, 12, 24, 48 and 72 h after BeG delivery. Six hours after each dacarbazine injection, levels of apoptosis were scored in the small intestine (Figure 5). Dacarbazine alone induced high levels of apoptosis with a mean of >60 apoptotic bodies per 50 half-crypts. However, between 1 and 6 h following BeG administration dacarbazine failed to induce apoptosis. This suppression was reversible, such that a normal apoptotic response was restored by 24 h. Thus, BeG administration reversibly inhibited dacarbazine-dependent apoptosis. The active DNA-damaging metabolite of dacarbazine is MITC, which is generated from dacarbazine in a cytochrome P450-dependent manner. In order to confirm that cytochrome P450 function was essential in eliciting a dacarbazine-dependent apoptotic response we used the P450 inhibitor metyrapone (25). Dacarbazine was administered 1 h following treatment with metyrapone (100 mg/kg i.p.). Metyrapone treatment significantly reduced the apoptotic response to dacarbazine (Figure 6), confirming that activation of the prodrug and the associated apoptotic response were P450 dependent ($P < 0.05$, Mann–Whitney $U$-test). These findings indicate that BeG administration blocks activation of the prodrug dacarbazine, possibly by compromising host P450 activity.

Discussion

We have directly addressed the ability of BeG to alter rates of apoptosis and mutation in the normal murine epithelium following exposure to a range of different types of damage. No induction of apoptosis was associated with BeG treatment. This excluded the possibility that either BeG itself or the depletion of AGT activity could result in the induction of apoptosis in the murine small intestine. Following treatment with BeG, and in the absence of detectable AGT activity, exposure to MNU, cisplatin and γ-irradiation all induced...
activity by depleting cellular pools of unmethylated AGT, so limiting the impact of BeG pretreatment (5, 26, 27). To address this possibility, temozolomide was administered at decreasing doses in the presence and absence of BeG. Similar levels of apoptosis were scored in both BeG-treated and BeG-untreated mice, suggesting that either ablation of AGT was occurring following exposure to very low levels of temozolomide or that BeG-mediated inactivation of AGT is irrelevant to the induction of apoptosis following alkylation damage. We further probed this question by analysing the effect of temozolomide administration upon AGT activity and established that low levels of temozolomide were effective in depleting AGT activity in the intestine. This is perhaps not surprising as resting levels within this tissue are relatively low. We also demonstrated a similar phenomenon for the liver, which is characterized by much higher basal AGT activity, although effective depletion was seen at the highest temozolomide dose used, 100 mg/kg.

Increasing doses of MNU increased the mutation frequency scored at the Dlb-1 locus. Since the principal mutagenic lesion generated by MNU is O6-methylguanine, ablation of AGT activity might be predicted to lead to an increase in Dlb-1 mutations. However, pretreatment of mice with BeG failed to elevate the mutation rate following MNU treatment. As with the effect of BeG treatment upon the induction of apoptosis, the observed failure to modify the mutation rate in the murine small intestine may be interpreted in several ways. First, AGT activity may not be relevant to protection against mutation in this tissue: other DNA lesions such as 3-methyladenosine could be responsible for these mutations. Second, exposure to the methylating agent alone may be sufficient to inactivate AGT, as suggested by our results following exposure to temozolomide. Third, it might be that in those cells in which Dlb-1 mutations normally arise, recovery of AGT activity occurs prior to fixation of the mutation by DNA replication. It seems likely that the second of these explanations is correct, although it should be remembered that we have only established effective AGT depletion at a single time point for temozolomide. Taken together, these results show that BeG pretreatment does not alter either the apoptotic response or mutation frequency in the normal epithelium of the murine small intestine.

A somewhat unexpected finding in the present study was that BeG administration blocks dacarbazine-induced apoptosis. It is reasonable to suggest that this occurs either because AGT activity is required by dacarbazine to induce apoptosis or because BeG is blocking the normal metabolism of dacarbazine.

apoptosis. However, the levels of apoptosis were similar to those observed in BeG-untreated controls. Thus, BeG treatment neither reduced nor augmented the prevalence of apoptosis in the intestine following application of a variety of DNA-damaging agents. Such a failure to modify the phenotype may have occurred because in vivo exposure to alkylating agents reduces AGT activity by depleting cellular pools of unmethylated AGT, so limiting the impact of BeG pretreatment (5, 26, 27). To address this possibility, temozolomide was administered at decreasing doses in the presence and absence of BeG. Similar levels of apoptosis were scored in both BeG-treated and BeG-untreated mice, suggesting that either ablation of AGT was occurring following exposure to very low levels of temozolomide or that BeG-mediated inactivation of AGT is irrelevant to the induction of apoptosis following alkylation damage. We further probed this question by analysing the effect of temozolomide administration upon AGT activity and established that low levels of temozolomide were effective in depleting AGT activity in the intestine. This is perhaps not surprising as resting levels within this tissue are relatively low. We also demonstrated a similar phenomenon for the liver, which is characterized by much higher basal AGT activity, although effective depletion was seen at the highest temozolomide dose used, 100 mg/kg.

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Fig. 4. The effect of temozolomide on AGT activity. AGT activity measured in fmol/mg protein scored in the liver (A) and in the small intestine (B) 6 h following a single i.p. injection of temozolomide. Resting values for intestine are reproduced from Toft et al. (13). Error bars represent SEM.

Fig. 5. The effect of BeG on dacarbazine-induced apoptosis. Following a single dose of BeG (60 mg/kg) at time 0, mice were injected with dacarbazine (150 mg/kg) at 0, 1, 6, 12, 24, 48 and 72 h after BeG. Six hours after each dacarbazine injection levels of apoptosis were scored in the small intestine. Each point represents data from three mice. Error bars represent SEM.

Fig. 6. The effect of metyrapone on dacarbazine-induced apoptosis. Bar chart of the prevalence of apoptotic bodies in the murine small intestine 6 h following 150 mg/kg dacarbazine or 100 mg/kg metyrapone. The prevalence of apoptotic bodies at 6 h in dacarbazine-treated mice which were pretreated with 100 mg/kg metyrapone 1 h prior to injection with dacarbazine is also shown (Met & Dac). Error bars represent SEM.
to an active alkylating agent. Dacarbazine is known to undergo oxidative N-demethylation to its active compound MITC (18,28,29). This metabolic activation is mediated through the action of cytochrome P450 enzymes (20,30). Two observations argue in favour of the possibility that BeG is blocking the normal metabolism of dacarbazine. First, temozolomide is able to induce apoptosis in the presence of AGT depletion (13). Thus, the inhibitory action of BeG cannot be arising subsequent to the generation of MITC since it is also the active compound formed when temozolomide undergoes spontaneous chemical transformation (19,21) (Figure 7). Second, one of the major routes of metabolism of BeG in mice involves oxidation to form O6-benzyl-8-oxoguanine (31), which occurs through the actions of aldehyde oxidase and the cytochrome P450 isozymes CYP1A2 and CYP3A4 (32,33). Dacarbazine is also metabolized by a number of different isozymes of P450, including CYP1A1, CYP1A2 and CYP2E1 (34). For both dacarbazine and BeG, the primary isozyme responsible for metabolism is CYP1A2. The possibility therefore arises that BeG metabolism may competitively deplete cytochrome P450 activity. Thus, BeG appears capable of inhibiting at least some of the demethylating ability of cytochrome P450 enzymes in the mouse liver. The consequences of this novel finding are likely to be significant in view of the central role of P450 enzymes in the metabolism of drugs and carcinogens.

There is increasing interest in the use of BeG clinically as an adjuvant to alkylating agent chemotherapy to overcome tumour resistance mediated by AGT and to potentiate the cytotoxic effects of chemotherapy (16,35). There are, however, a number of uncertainties concerning the clinical benefits of BeG. First, alkylating agents themselves are capable of inactivating AGT activity by depleting cellular pools (5,27). We show here that this can be an effective mechanism for suppressing AGT activity both in the normal cells of the small intestine and in the liver of mice. There are, however, several lines of evidence to suggest that the use of alkylating agents to suppress AGT activity can be ineffective or even inappropriate, either because of failure to increase the therapeutic index or because of dose-limiting toxicity (27). Thus, although alkylating agents can reduce the activity of AGT, the available data indicate BeG administration to be the most effective and least toxic method to achieve this. Second, BeG has been reported to increase mutation rates, chromosome aberrations and toxicity in normal cells treated with methylating or chloroethylating agents (36–38). In contradiction to these reports, we do not observe any effect upon in vivo mutation rate in these studies. Third, from the data presented here it is clear that in the murine small intestine AGT depletion does not elevate in vivo apoptotic levels following methylating DNA damage. This result has clear positive implications for the therapeutic use of BeG, in that BeG administration fails to augment cell death within normal tissues. This result may directly follow from the demonstrated ability of alkylating agents to partially suppress AGT activity and may simply reflect the low resting levels of AGT in the murine intestine. Finally, and perhaps most significantly, these data show that BeG can alter the metabolism of drugs or carcinogens, exemplified here by activation of the prodrug dacarbazine, and may therefore be a cause of unexpected adverse drug reactions if used clinically.

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to evaluate the role of this protein in protection against carcinogenic and therapeutic alkylating agents. 


