Lack of Evidence for a Polymorphism at Codon 160 of Human $O^6$-Alkylguanine-DNA Alkyltransferase Gene in Normal Tissue and Cancer

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ABSTRACT

$O^6$-benzylguanine (BG) is a potent, specific inactivator of the DNA repair protein $O^6$-alkylguanine-DNA alkyltransferase (AGT), which enhances sensitivity to nitrosoureas in cells and tumor-bearing animals. BG is presently undergoing clinical trials for development as an agent to enhance the therapeutic index of alkylating agent chemotherapy. It has been reported that a polymorphism exists in the human $agt$ gene, with about 15% of the Japanese population having arginine at codon 160 instead of glycine on the polypeptide (Y. Imai et al., Carcinogenesis, 16: 2441–2445, 1995). The resultant mutant AGT protein is equally effective against both methylated DNA as compared with wild type protein. However, this mutant AGT protein was less sensitive to inactivation by BG with a 20-fold increase in the ED$_{50}$ value. This observation raised the possibility that a subpopulation of patients may be resistant to BG due to a single base change. We have demonstrated that this alteration also reduces the sensitivity to $O^6$-benzyl-8-oxoguanine, an equally potent, yet much longer-lived human metabolite of BG. To test the possibility that this germline mutation of the $agt$ gene might explain resistance to BG and $O^6$-benzyl-8-oxoguanine of patients on our Phase I clinical trials, we evaluated the DNA from lymphocytes of 18 patients. The G160R mutation was not found in any of the 18 patients. To determine the frequency of this mutation in the United States population, DNA from 181 healthy individuals were investigated and, again, the mutation was not observed in this cohort. Therefore, if the mutation exists, it is in statistically $<1.6\%$ of the United States noncancerous population. To investigate the possibility that this mutation might be somatic, we evaluated genomic DNA samples from 94 human primary cancers of four different histological subtypes (brain, colon, esophageal, and head and neck). Again, none were found to have the G160R mutation.

INTRODUCTION

The DNA repair protein AGT (MGMT, EC 2.1.1.63) has been implicated in the recognition and repair of alkylator-induced DNA damage introduced by chloroethylnitrosoureas (BCNU and CCNU) and methylating agents (temozolomide, dacarbazine, procarbazine, and streptozotocin; Ref. 1). The presence of the AGT protein imparts resistance to chemotherapeutic alkylating agent damage by removing toxic lesions formed at the $O^6$-position of guanine. Chloroethylnitrosourea cross-links are prevented from forming either by removal of the chloroethyl lesion from the $O^6$-position before rearrangement, or reaction with the intermediate (1,$O^6$-ethanoguanine) to form a cross-link between DNA and the repair protein (2–7). $O^6$-methylguanine induced by methylating agents is repaired by removal of the methyl group from the $O^6$-position of guanine, leaving guanine intact in DNA. The AGT protein is inactivated in the repair process. Efforts to overcome resistance by inactivation of the AGT led to the development of low molecular weight substrates for the protein, including BG. BG is a potent, specific inactivator of the AGT protein that enhances the sensitivity of tumor cell lines and tumor-bearing animals to alkyl-nitrosoureas (6, 8–10). Inactivation by reaction of the cysteine within the active site of the protein with benzyl group results in rapid degradation of the protein in intact cells and in cell-free extracts (11). Clinical trials of the combination of BG with BCNU are presently underway (12–14).

Recently, Imai et al. (15) reported a germline mutation at codon 160 in exon 5 of the $agt$ gene converting glycine to arginine. The polymorphism was present in about 15% of the Japanese population under study (15). Although the mutation is only 15 amino acids distant from the active site cysteine, the mutation does not affect the activity against $O^6$-methylguanine within DNA. However, the mutant protein was significantly less sensitive to inactivation by BG with at least a 20-fold increase in the ED$_{50}$ value (8). These results indicate that it is likely that

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3 The abbreviations used are: AGT, $O^6$-alkylguanine-DNA alkyltransferase; BG, $O^6$-benzylguanine; 8-oxo-BG, $O^6$-benzyl-8-oxoguanine; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea.
a subpopulation of patients resistant to BG may exist. Previous studies by Cron et al. (16, 17) using site-directed mutagenesis revealed that point mutations at other positions in exon 5 or combinations of these mutations produced proteins even more resistant to inactivation by BG, several completely resistant up to 1 mM of BG. It has been suggested that these mutations change the structure of the protein in such a way as to decrease hydrophobicity or the size of the active site pocket no longer allowing the benzyl group to fit. Expression of the double mutant, G156A and P140A protein in CHO cells rendered cells resistant to BCNU and, as expected, BG was much less effective in restoring sensitivity to BCNU (18, 19).

The finding of a “naturally occurring” variant of the human AGT with decreased sensitivity to BG has dramatic implications for the clinical development of this drug (20). It suggests that a significant fraction of patients may show a decreased response to BG and should be treated with a higher dose of drug or an AGT inactivator effective against the mutant protein. We have observed significant interpatient variability in AGT inactivation in the peripheral blood mononuclear cells of patients enrolled on the Phase I study of BG. BG is oxidized in humans to an equally potent, yet much longer-lived AGT-inactivator, 8-oxo-BG (12, 14). Higher concentrations of 8-oxo-BG are found in human plasma than concentrations of BG (14). If this “mutant” protein is present to a significant extent in the population, then sensitivity to 8-oxo-BG may be more important clinically than sensitivity to the parent drug, BG.

The aim of this work was 4-fold: (a) to determine the sensitivity of the G160R mutant protein to 8-oxo-BG; (b) to determine whether interpatient variability observed in the Phase I study of BG was a result of differences in the sensitivity of AGT to BG due to the G160R mutation; (c) to determine whether the G160R mutation was present in the United States population; and (d) to determine the extent to which G160R was present in human tumors (i.e., colon, head and neck, esophageal, and brain tumors).

MATERIALS AND METHODS

Materials. The oligonucleotides 5'-GTCCCCCATCCT-CATCCCCGTG-3' and 5'-CTCAGTTTCGGCCAGGC-3' were synthesized by Genosys Biotechnologies, Inc. (The Woodlands, TX). The Xpress Protein Purification System was from Invitrogen Corp. (San Diego, CA). The human AGT cDNAs (wild type and mutant) were kindly provided by Dr. Anthony E. Pegg (Pennsylvania State University College of Medicine, Hershey, PA). The restriction endonuclease BspE I deoxyribonucleotide triphosphates, and Escherichia coli strain JM109 were purchased from New England Biolabs (Beverly, MA). Histoplasma-1077 was purchased from Sigma Chemical Co. (St. Louis, MO). RPMI 1640 was obtained from Mediatech (Herndon, VA).

Isolation of Lymphocytes. Blood (40 ml) was collected from noncancerous volunteers in EDTA-containing tubes. Immediately after collection, an equal volume of RPMI medium was added and the diluted blood was layered on Ficoll-Paque (Histoplasma 1077). After centrifugation at 400 × g for 30 min, the lymphocyte layer was removed and resuspended in 15 ml of PBS and centrifuged at 250 × g for 10 min at room temperature. RBCs were lysed by the addition of 6 ml of deionized water for 15 s, after which 2 ml of 3.6% NaCl and 15 ml of PBS were added. The samples were centrifuged at 250 × g for 10 min. Final pellets were resuspended in 50 mM Tris (pH 7.5), 0.1 mM EDTA, and 5 mM DT.

Purification of Alkyltransferase. The open reading frame of the human alkyltransferase was subcloned into pQE30 and expressed in E. coli JM109. The expressed protein is tagged at the amino terminus with a histidine track (18). The addition of the (His)n tag to the amino terminus of the protein by the pQE30 vector to facilitate purification does not affect the properties of the protein (20). The growth of E. coli culture and protein purification were as previously described (20), except that the concentration was increased.

Assay of Alkyltransferase Activity. Before assaying for AGT activity, increasing concentrations of BG or 8-oxo-BG were incubated in 50 mM Tris (pH 7.5), 0.1 mM EDTA, and 5 mM DT buffer with pure alkyltransferase protein (7 μg wild type or 14 μg G160R mutant) and calf-thymus DNA (50 μg) or glycerol (20%) for 30 min at 37°C. Under these conditions, the AGT activity was completely stable with <5% loss of activity in the 30-min incubation in the absence of AGT inhibitor. The assay for alkyltransferase activity was performed as previously described (9, 21). Briefly, alkyltransferase activity was measured as removal of 32P-[H]methylguanine from a [3H]-methylated DNA substrate (5.9 Ci/mmol) after incubation with extract or pure protein at 37°C for 30 min. The DNA was precipitated by adding ice-cold perchloric acid (0.25 M) and hydrolyzed by the addition of 0.1 M HCl at 70°C for 30 min. The modified bases were separated by reverse-phase high performance liquid chromatography with 0.05 M ammonium formate (pH 4.5) containing 7.5% methanol. Protein concentration of cell extract was determined by the method of Bradford (22), and the samples were expressed as nmol of 32P-methylguanine released from the DNA substrate/mg of protein.

PCR Product Digestion and Gel Electrophoresis. Genomic DNA was purified from whole blood or tissue using the Puregene DNA isolation kit (Gentra Systems Inc., Minneapolis, MN). After isolation, DNA was diluted 10 times with distilled water and stored at −70°C until use. PCR was carried out in a total volume of 20 μl consisting of 250 μM each of the deoxyribonucleotide triphosphates, 2 μl Buffer II [50 mM KC1, 10 mM Tris-HCl (pH 8.3), 1.25 mM MgCl2, 0.1 μM each of the primers, 1 μl (−50 ng) of the chromosomal DNA, and 1 unit of AmpliTaq DNA Polymerase Gold (Perkin-Elmer Corp., Branchburg, NJ)]. Control reaction mixtures contained wild-type or mutant agt cDNA in place of chromosomal DNA. Forty-step cycles of amplification were performed in a Perkin-Elmer 480 thermocycler (92°C, 1 min; 61°C, 1 min; 72°C, 20 s), with an initial pre-denaturation at 94°C for 9 min. PCR products (2 μl) were then digested with BspE I (an Mro I isoschizomer). The digested reaction was run on a 1.5% (w/v) agarose gel containing 1×10,000 (v/v) of SYBR Green I (Molecular Probes, Eugene, OR), along with digested PCR product of positive (agt cDNA with the G160R mutation) and negative (wild-type agt cDNA) controls, and visualized under 360 nm UV light. The distribution of the mutation among individuals was assumed binomial, and the 95% confidence intervals for mutation rate were calculated using STATA 5.0 (College Station, TX).
**Table 1** Sensitivity of wild type and G160R mutant proteins to BG and 8-oxo-BG.

<table>
<thead>
<tr>
<th>Protein</th>
<th>ED₅₀ (μM) + Glycerol</th>
<th>ED₅₀ (μM) + 50 μg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.4 ± 0.2</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>G160R</td>
<td>9.8 ± 6.0</td>
<td>4.0 ± 1.1</td>
</tr>
<tr>
<td>Fold-difference</td>
<td>24.5</td>
<td>20</td>
</tr>
</tbody>
</table>

**Fig. 1** Detection of G160R mutation by PCR and BspEI digestion. Lanes are labeled by individual chromosomal DNA used as a template in PCR. Wild type and mutant cDNAs were used as controls both in PCR and BspEI digestion. M, molecular weight marker; WT, wild type; MT, G160R cDNA; T1 and T2, genomic DNA from two tumors; S22 and S23, genomic DNA from two normal volunteers; +, PCR product digested with BspEI; −, BspEI not added.

**RESULTS**

**AGT Sensitivity to BG and 8-oxo-BG.** Human wild type and G160R mutant AGT proteins were expressed in E. coli JM109 from the pQE30 vector and purified to homogeneity. AGT activity was determined on wild type and G160R mutant AGT proteins. Consistent with previous results, the mutation at glycine 160 did not affect the level of expression or the specific activity of the purified preparation against methylated DNA. Equivalent amounts of proteins were used to determine the sensitivity of wild type and G160R mutant protein to BG and 8-oxo-BG. As illustrated in Table 1, the conversion of glycine 160 to arginine residue rendered the protein much more resistant than control AGT, increasing the ED₅₀ by 20-fold for BG in the presence of DNA. These results are consistent with previous studies demonstrating a decrease in sensitivity of the “G160R mutant” AGT to BG (20). We now demonstrate an equivalent decrease in sensitivity to 8-oxo-BG (23.5-fold).

**PCR Product Digestion and Sequencing.** The wild type and G160R agt cDNA and the genomic DNA PCR products from the brain tumor and lymphocyte from a healthy volunteer, purified from agarose gel (Life Technologies, Inc., Gaithersburg, MD), were sequenced by cycle sequencing using the ABI prism rhodamine terminator cycle sequencing reaction kit, and the sequence data were generated on the ABI 377 sequencer from PE Applied Biosystems (Foster City, CA).

**DNA Sequencing.** The wild type and G160R agt cDNA and the genomic DNA PCR products from the brain tumor and lymphocyte from a healthy volunteer, purified from agarose gel (Life Technologies, Inc., Gaithersburg, MD), were sequenced by cycle sequencing using the ABI prism rhodamine terminator cycle sequencing reaction kit, and the sequence data were generated on the ABI 377 sequencer from PE Applied Biosystems (Foster City, CA).

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**PCR Product Digestion and Sequencing.** The G160R mutation is caused by a point mutation in which the guanine is substituted for adenine in exon 5 of the agt gene. The sequence is then changed from TCCGGA to TCCAGA, resulting in loss of the BspEI site. Primers were designed such that the wild-type exon 5 yielded the expected digestion product of BspEI as 145 bp and 66 bp, and the mutant sequence that was refractory to cleavage resulted in a 211-bp PCR product. A representative gel of the reaction products of two normal individuals, wild-type cDNA, and G160R mutant cDNA is shown in Fig. 1. Note that mutation in a single allele (heterozygous) would result in partial digestion with three fragments as 211 bp, 145 bp, and 66 bp, respectively.

**Analysis of DNA from Patients Enrolled on the BG Trial.** It was considered that the mutation at G160R might explain the insensitivity of lymphocytes in patients enrolled on our Phase I trial to BG. The repletion kinetics of AGT activity in lymphocytes of five patients treated with 40 mg/m² BG for up to 72 h after iv infusion of drug is illustrated in Fig. 2. Four of the patients were equally sensitive to BG, whereas one individual was much less sensitive to the AGT-inactivating effects of BG and 8-oxo-BG, an equally potent, yet longer-lived metabolite in plasma. It was considered that this individual might be less sensitive due to a G160R mutation. However, none of the five patients evaluated had a G160R germ-line mutation, which would result in a resistant form of AGT protein, nor did an additional 13 patients.

**Table 2** Frequency of G160R mutation in normal tissue of noncancerous individuals.

<table>
<thead>
<tr>
<th>Ethnic origin</th>
<th>n</th>
<th>Wild type</th>
<th>Mutant</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caucasian</td>
<td>107</td>
<td>107</td>
<td>0</td>
<td>2.8%</td>
</tr>
<tr>
<td>African American</td>
<td>37</td>
<td>37</td>
<td>0</td>
<td>7.8%</td>
</tr>
<tr>
<td>Hispanic</td>
<td>13</td>
<td>13</td>
<td>0</td>
<td>20.6%</td>
</tr>
<tr>
<td>Indian Subcontinent</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>Asian/Pacific Islander</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>Japanese</td>
<td>16</td>
<td>16</td>
<td>0</td>
<td>17.1%</td>
</tr>
<tr>
<td>Total</td>
<td>181</td>
<td>181</td>
<td>0</td>
<td>1.6%</td>
</tr>
</tbody>
</table>

*CI, confidence interval; NA, not applicable.*
Analysis of DNA from Healthy Controls. Because only 15% of the Japanese population were shown to have this mutation, we reasoned that there may be ethnic differences in the degree to which the polymorphism existed. A total of 181 healthy control individuals of various ethnic backgrounds were evaluated and none were shown to contain a mutation that would result in a G160R mutant protein, because the BspI restriction site was not altered. Zero mutations of 181 samples yield a 95% upper bound for the underlying mutation frequency of 1.6% (Table 2). Thus, it is very likely that if the mutation exists, the frequency is <1.6% in the United States population.

Analysis of DNA from Tumor Specimens. To test whether this mutation occurs in tumor cells from patients with diverse tumors, 94 tumor samples were evaluated. Again, no mutations of 94 tumors were identified. The types of tumors analyzed included colon (n = 28), head and neck (n = 35), brain (n = 29), and esophagus (n = 2). Zero mutations of 94 tumors yield a 95% upper bound for the underlying mutation frequency among tumors of these types of <3.1%. An additional 28 normal colon tissues were evaluated and, again, the BspI restriction site was not altered in any of the samples. The 29 brain tumors analyzed included 14 glioblastoma multiformes (adult), 9 ependymomas (pediatric), and 5 astrocytomas (pediatric). One of these brain tumors represented a patient who had received a dose of 100 mg/m² BG and was much less sensitive at 1.6%. One of these brain tumors represented a patient who had received a dose of 100 mg/m² BG and was much less sensitive to drug (23). It was thought that this patient may have a somatic mutation resulting in tumor AGT protein resistant to BG, however, this patient exhibited wild type protein as determined by PCR analysis. To rule out the possibility that other mutations in exon 5 could explain the lack of sensitivity to drug, the DNA was extracted and sequenced. The 211-bp sequence was consistent with wild type.

DISCUSSION

Our results clearly show that the presence of the basic arginine residue at position 160 in place of glycine, reported in 15% of the Japanese population, substantially reduces the ability of the protein to act on both BG and 8-oxo-BG. It was expected that the interpatient variability of AGT inactivation in our Phase I clinical trial might be explained by this polymorphism. This mutation was not identified in 18 Phase I trial patients, nor in 181 normal, noncancerous individuals, suggesting that it is extremely unlikely to explain interpatient variability to BG. Furthermore, this mutation was not identified in 94 tumor samples.

The 181 healthy individuals represent a variety of ethnic groups, including Japanese (Table 2). Our data based on a limited sampling of Japanese (n = 16) does not rule out the possibility that the polymorphism exists to a significant extent in this population. One explanation could be in the selection of individuals. We evaluated DNA from healthy individuals, whereas Imai et al. (15) used DNA samples from young patients with adult-type cancers. It is possible that this subgroup of patients exhibits the G160R mutation, although the mutation was also evaluated in noncancerous Japanese adult patients at a frequency of 10.7% (15). It is possible that there are ethnic differences in the degree of expression of the mutation.

Differences in AGT depletion/repletion kinetics after BG administration in patients enrolled on our Phase I trial must be explained by reasons other than the G160R mutation. It is possible that differences in sensitivity of patients to BG are due to differences in the metabolism of BG (14) or differences in the rate of resynthesis of AGT in lymphocytes from individuals, or other as yet unidentified sequence variants. Our data are in agreement with that of Gerson et al.4 who evaluated the phenotype of 40 individuals. In this study, the extent to which BG depleted AGT in extracts prepared from these individuals indicated no more than a 2-fold difference in sensitivity to drug.

Recently Deng et al. (24) identified a novel missense polymorphism at 143 in exon 5 converting isoleucine (ATC) to valine (GTC). This was linked to a second alteration at codon 178, which converts lysine (AAG) to arginine (AGG). The codon 143/178 was found in 2% of esophageal patients in China and in 21% of noncancer Caucasian subjects. It is not known whether this mutation confers resistance to BG or to 8-oxo-BG. A recent study by Otsuka et al. (25) reported three variants of the alkyltransferase gene detected after evaluating 225 healthy Japanese and 173 colorectal cancer patients. One variant had a C→T transition at nt 262, thus causing a single amino acid change (Leu 84→Phe) combined with an additional silent C→T transition at nt 171. The second variant had G→C transversion at nt 207, which would cause Trp65→Cys. Both variants were in exon 3. The third variant had a silent C→A transition at nt 579. The allele frequencies of the first and second variant were 0.162 and 0.002, respectively. Further characterization with regard to the resultant mutant proteins has not been carried out. In a more recent effort to correlate agt gene mutations and the occurrence of esophageal cancer in northern China, Wang et al. (26) investigated 40 families with a high incidence of esophageal cancer for mutations in the agt gene. Point mutations were found in seven esophageal cancer tissues (three cases of Ala121→Thr/Glu, two cases of Asn123→Val, one case of Gly132→Arg, and silent mutations occurred at codons 111 and 120). None of these were observed in their paired normal tissues.

Although we found no G160R mutations in the 94 tumor samples, it is possible that agt gene mutations may arise that result in proteins resistant to BG therapy. Expression of a mutant protein may be selected for following BG and alkylating agent treatment. Thus, our results would place emphasis on evaluating tumors from patients highly resistant to this therapy with regard to the sequence of the agt gene.

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REFERENCES


