Efficient Repair of O\textsuperscript{6}-Ethylguanine, but not O\textsuperscript{4}-Ethylthymine or O\textsuperscript{2}-Ethylthymine, Is Dependent upon O\textsuperscript{6}-Alkylguanine-DNA Alkyltransferase and Nucleotide Excision Repair Activities in Human Cells

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Abstract

The formation and persistence of O\textsuperscript{6}-ethylguanine, O\textsuperscript{4}-ethylthymine, and O\textsuperscript{2}-ethylthymine were quantitated in the genomic DNA of human lymphoblasts exposed to 1.0 mM N\textsuperscript{2}-ethyl-N\textsuperscript{2}-nitrosoethane using immunoslot-blot. The three cell lines used included one which lacks O\textsuperscript{6}-alkylguanine-DNA alkyltransferase, one deficient in nucleotide excision repair, and a third which is competent in both of these repair pathways. The activity of O\textsuperscript{6}-alkylguanine-DNA alkyltransferase was further modulated with O\textsuperscript{2}-benzylation, a specific inhibitor of this protein. Repair of the O\textsuperscript{2}-ethylated thymines was slow and not related to either DNA repair phenotype. O\textsuperscript{6}-Ethylguanine was repaired with a half-life of about 8 h in cells which expressed both O\textsuperscript{6}-alkylguanine-DNA alkyltransferase and nucleotide excision repair functions. Cells expressing O\textsuperscript{6}-alkylguanine-DNA alkyltransferase activity but lacking nucleotide excision repair showed only slow repair of O\textsuperscript{6}-ethylguanine (half-life of O\textsuperscript{6}-ethylguanine, 43 h), while cells lacking the alkyltransferase showed little or no repair of O\textsuperscript{6}-ethylguanine regardless of nucleotide excision repair activity (half-lives of O\textsuperscript{6}-ethylguanine, 53 to >100 h). We conclude that O\textsuperscript{6}-alkylguanine-DNA alkyltransferase and nucleotide excision repair cooperate in the repair of O\textsuperscript{6}-ethylguanine in human cells.

Introduction

Chemical modification of DNA by simple alkylating compounds such as the nitrosoureas can result in cytotoxicity and mutagenesis in human cells (1, 2). ENU\textsuperscript{2} is a potent ethylating mutagen and carcinogen that can react with all of the purine and pyrimidine nitrogens and oxygens as well as the phosphate oxygens in DNA (3). Two reaction products, O\textsuperscript{6}-ethylguanine and O\textsuperscript{4}-ethylthymine, have been shown to be efficient promutagenic adducts in vivo, and O\textsuperscript{6}-ethylthymine may direct mis-incorporation by DNA polymerases as well (4, 5). Repair of DNA adducts can reduce the toxicity and mutagenesis associated with exposure to alkylating agents (1, 2). An important repair protein is AGT, which can repair O\textsuperscript{6}Gua lesions in DNA by directly transferring the methyl adduct to a cysteine within its own sequence (6). Less is known about the repair of O\textsuperscript{4}Gua, O\textsuperscript{4}TThy, and O\textsuperscript{2}Thy lesiions in mammalian cells. However, human cells derived from patients with xeroderma pigmentosum complementation group A (XPA), which lack NER capability, are highly sensitive to the toxic and mutagenic effects of ENU (1, 2), suggesting an important role for NER in the repair of ethyl adducts.

We have used the technique of immunoslot-blot (7) to measure the levels of O\textsuperscript{6}Gua, O\textsuperscript{4}Thd, and O\textsuperscript{2}Thd in human lymphoblastoid cells over a 24-h period following exposure to 1.0 mM ENU. By using cell lines with different DNA repair phenotypes and by depleting cells of AGT activity with a specific inhibitor, BzGua (8), we have been able to monitor the persistence of adducts in populations expressing each of the four possible combinations of AGT and NER activity. Our results indicate that only cells expressing both AGT and NER are able to efficiently remove O\textsuperscript{6}Gua from genomic DNA. Repair of the O\textsuperscript{4}-ethylated thymines was slow and did not correlate with either of these DNA repair pathways.

Materials and Methods

Cells and ENU Exposures. The human B-lymphoblastoid cell lines GM0130B (N cells; “normal”) and GM2250C (X cells, “excision-repair deficient”) were obtained from the National Institute of General Medical Sciences (Camden, NJ). The DNA repair characteristics and conditions for growth of these cells and of TK6 (A cells, “alkyltransferase deficient”) have been described previously (2). Exponentially growing cultures were diluted to a density of 4 × 10\textsuperscript{5} cells/ml. Each culture received either a 25 mM solution of BzGua (provided by Drs. Anthony Pegg and Robert Moschel) in dimethyl sulfoxide for a final concentration of 25 μM, or an equivalent volume of dimethyl sulfoxide, and was incubated at 37°C for 2 h. ENU (Sigma Chemical Co., St. Louis, MO) was prepared as a 1 mM stock solution in dimethyl sulfoxide and delivered for a final concentration of 0.1 or 1 mM. Cells were incubated at room temperature for 1 h and then centrifuged (1500 rpm for 3 min), placed in fresh media containing either 25 μM BzGua or dimethyl sulfoxide, and incubated at 37°C. Aliquots were removed from these cultures 0, 6, and 24 h after the end of ENU exposure. Population growth was monitored by measuring cell density with a Coulter Counter (Coulter Electronics, Hialeah, FL). These aliquots were centrifuged, washed with 10 ml phosphate-buffered saline, and frozen as cell pellets at −80°C for future analysis.

DNA Adduct Measurements. The content of O\textsuperscript{6}Gua, O\textsuperscript{4}Thd, and O\textsuperscript{2}Thd in cellular genomic DNA was measured by immunoslot-blot according to the procedure of Nehls et al. (7). Approximately 10\textsuperscript{7} washed cells were resuspended in 3.5 ml lysis buffer (Applied Biosystems, Emeryville, CA). RNA was digested with 50 units RNase A (Sigma) and 100 units of RNase T\textsubscript{2} (Boehringer-Mannheim, Indianapolis, IN) at 37°C for 1 h; then protein was digested with 40 units proteinase K (Applied Biosystems) at 37°C for 1 h. DNA was isolated by phenol/chloroform extraction and isopropanol alcohol precipitation using an Applied Biosystems 340A Nucleic Acids Extractor. DNA samples were resuspended in 100 μl buffer (10 mM Tris-1 mM EDTA, pH 7.4), and the yield was determined by high performance liquid chromatography analysis. DNA samples and standards (3 μg/μl), were immobilized on BA-85 nitrocellulose (Schleicher and Schuell, Keene, NH). The monoclonal mouse antibodies EM-21 (anti-O\textsuperscript{6}Gua) and EM-41 (anti-O\textsuperscript{4}Thd) were provided by Dr. Gertrud Eberle, and

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2 The abbreviations used are: ENU, N\textsuperscript{2}-ethyl-N\textsuperscript{2}-nitrosoethane; AGT, O\textsuperscript{6}-alkylguanine-DNA alkyltransferase; NER, nucleotide excision repair; O\textsuperscript{6}Gua, O\textsuperscript{4}Gua, O\textsuperscript{2}Gua; O\textsuperscript{6}Thd, O\textsuperscript{4}Thd, O\textsuperscript{2}Thd; O\textsuperscript{6}ethylthymine, O\textsuperscript{4}ethylthymine, O\textsuperscript{2}ethylthymine; O\textsuperscript{6}Gua; O\textsuperscript{6}ethyldeoxyguanosine; O\textsuperscript{2}Gua; O\textsuperscript{2}ethyldesoxycytidine; O\textsuperscript{4}Thd; O\textsuperscript{4}ethylthymidine; O\textsuperscript{2}Thd; O\textsuperscript{2}ethylthymidine; dGua, deoxyguanosine; N cells, GM0130B; X cells, GM2250C; A cells, TK6; BzGua, O\textsuperscript{6}benzylguanine; XP, xeroderma pigmentosum.

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the polyclonal rabbit antibody TB3 (anti-O\textsuperscript{6}-ethylG) was provided by Dr. Altaf Wani. Specific adduct binding by the first antibody and subsequent washes were performed as described (7). Detection of the first antibody was performed using biotinylated anti-mouse or anti-rabbit immunoglobulin (Amersham, Arlington Heights, IL) and streptavidin-biotinylated horseradish peroxidase (Amersham), with visualization by enhanced chemiluminescence (Amersham) and Kodak XAR 5 X-ray film. Exposed films were analyzed by scanning densitometry using an Ultroscan XL (Pharmacia LKB, Piscataway, NJ).

Adduct concentrations were expressed as number of adducts per unmodified base. At the 6- and 24-h time points, adduct concentrations were multiplied by the measured growth of the alkylated populations to correct for adduct dilution due to DNA replication.

**Results**

Among the 6 ENU exposure groups tested, only N cells, which express both AGT and NER, demonstrated efficient repair of O\textsuperscript{6}-ethylG (Fig. 1A; Table 1). Each of the other 5 exposure groups showed little or no repair of this lesion. These 5 groups include three phenotypes of DNA repair: (a) cells which have reduced AGT activity either due to lack of AGT expression (A cells with or without BzGua) or due to AGT depletion by BzGua (N cells with BzGua); (b) cells deficient in NER, but expressing AGT (X cells); and (c) cells deficient in both AGT and NER (X cells with BzGua).

In N cells, O\textsuperscript{6}-ethylG was removed with an apparently constant half-life of approximately 8.1 h in the first 24 h following alkylation, while each of the other groups had half-lives for repair of O\textsuperscript{6}-ethylG in excess of 40 h (Table 1). The long half-lives for O\textsuperscript{6}-ethylG repair in cells lacking AGT indicate that AGT is necessary for the efficient removal of O\textsuperscript{6}-ethylG. The fact that the half-life for the repair of O\textsuperscript{6}-ethylG increases over 5-fold with the removal of NER capacity, from 8.1 h in N cells to 43.4 h in X cells, shows that NER function is also necessary for the efficient repair of O\textsuperscript{6}-ethylG. Not surprisingly, X cells treated with BzGua, which possess neither repair function, showed no detectable repair of O\textsuperscript{6}-ethylG. These results demonstrate that human cells which express both AGT and NER can effectively remove O\textsuperscript{6}-ethylG from genomic DNA. Cells which lack either AGT, NER, or both repair systems do not efficiently repair O\textsuperscript{6}-ethylG.

The repair of O\textsuperscript{6}-ethylThd in each of the cell lines was seen to occur only at a very slow rate (Fig. 1B). Due to the variability associated with these measurements, it was possible to conclude only that the half-life of active repair was greater than 20 h. No correlation was detected between either AGT or NER and the repair of O\textsuperscript{6}-ethylThd. There appeared to be some repair of O\textsuperscript{6}-ethylThd (Fig. 1C), but little difference was seen between the exposure groups in the repair of this adduct, and no correlation was seen between the removal of this adduct and either AGT or NER.

**Discussion**

We have examined the levels of O\textsuperscript{6}-ethylG, O\textsuperscript{6}-ethylThd, and O\textsuperscript{6}-ethylThd by the technique of immunoslot-blot (7) in the genomic DNA of three human lymphoblastoid cell lines exposed to 1.0 mM ENU. Adduct concentrations are expressed as the fraction of the original concentrations. □, N cells; ○, X cells. △, A cells. ■, ○, △, A cells treated with ENU only; □, ○, △, ENU and BzGua. Bars, SD. For clarity, error bars for X cells and X with BzG have been shifted left, while those for A cells and A with BzG have been shifted right. A, O\textsuperscript{6}-ethylG. Level at 0 h = 6.6 ± 2.6 × 10\textsuperscript{-6} O\textsuperscript{6}-ethylG/dGuo. B, O\textsuperscript{6}-ethylThd. Level at 0 h = 0.9 ± 0.4 × 10\textsuperscript{-6} O\textsuperscript{6}-ethylThd/dThd. C, O\textsuperscript{2}-ethylIT. Level at 0 h = 2.1 ± 0.9 × 10\textsuperscript{-6} O\textsuperscript{2}-ethylIT/dTtd.

Fig. 1. Repair of DNA ethyl adducts in the genomic DNA of human lymphoblasts exposed to 1.0 mM ENU. Adduct concentrations are expressed as the fraction of the original concentrations. □, N cells; ○, X cells. △, A cells. ■, ○, △, A cells treated with ENU only; □, ○, △, ENU and BzGua. Bars, SD. For clarity, error bars for X cells and X with BzG have been shifted left, while those for A cells and A with BzG have been shifted right. A, O\textsuperscript{6}-ethylG. Level at 0 h = 6.6 ± 2.6 × 10\textsuperscript{-6} O\textsuperscript{6}-ethylG/dGuo. B, O\textsuperscript{6}-ethylThd. Level at 0 h = 0.9 ± 0.4 × 10\textsuperscript{-6} O\textsuperscript{6}-ethylThd/dThd. C, O\textsuperscript{2}-ethylIT. Level at 0 h = 2.1 ± 0.9 × 10\textsuperscript{-6} O\textsuperscript{2}-ethylIT/dTtd.

plus the rate of repair in N cells with BzGua (the rate of removal by NER alone) should equal the rate of repair in N cells (the rate of removal by AGT and NER together). The calculation of a combined rate of repair for N cells with BzGua and X cells produces a hypothetical half-life for O\textsuperscript{6}-ethylG repair of

\[
\frac{1}{53 + 43.4} = 24 h
\]

which is in great excess of the 8.1-h half-life seen in N cells. These results indicate that, although AGT and NER may each have some ability to repair O\textsuperscript{6}-ethylG independently, the two systems must interact to efficiently remove O\textsuperscript{6}-ethylG from the chromosomal DNA of human cells.

At the measured alkylation level of about 7 × 10\textsuperscript{-8} O\textsuperscript{6}-ethylG/oGU which occurs in these cells following exposure to 1.0 mM ENU, the total number of O\textsuperscript{6}-ethylG adducts in each cell would be about 1.5 × 10\textsuperscript{4}. N cells contain about 10\textsuperscript{4} AGT molecules/cell, while X cells express slightly higher levels of AGT, by a factor of approximately 1.4 (2). Thus, while the adducts formed could possibly saturate the available AGT molecules, this is not likely to be the cause of the differences observed in repair. If AGT levels were being depleted by the removal of O\textsuperscript{6}-ethylG, the initial rates of repair would be similarly rapid in both cell lines and would slow at later times with the disappearance of AGT (9). Instead, N cells appeared to remove O\textsuperscript{6}-ethylG with a
This means that less than one-half of the constitutive AGT constant half-life, as is evidenced by the straight line seen in the semilog plot of adduct concentration versus time (Fig. 1A). The greater than 85% repair of O\textsubscript{6}edGuo in 24 h in N cells would correspond to approximately $1.3 \times 10^4$ adducts, which would more than deplete the cellular AGT molecules in the absence of resynthesis. Active induction of AGT expression has not been shown to occur in human cells (9); therefore up-regulation of AGT expression by ENU exposure in N cells is unlikely to explain the differences on O\textsubscript{6}edGuo repair. In contrast to the N cells, X cells do not show rapid removal of O\textsubscript{6}edGuo at any time over the 24 h following alkylation. Approximately 30% of the O\textsubscript{6}edGuo was repaired within 24 h in these cells, which would correspond to roughly $5 \times 10^3$ adducts. This means that less than one-half of the constitutive AGT molecules in these cells would have been used and indicates that AGT in the X cells is unable to efficiently remove O\textsubscript{6}edGuo from the chromosomal DNA.

Several explanations can be put forward to address the observed patterns of O\textsubscript{6}edGuo repair. One is that AGT activity is the chief mechanism for the removal of O\textsubscript{6}edGuo adducts from DNA, but that the protein needs help gaining access to adducts which lie within the complex structure of chromatin. This explanation is attractive, since purified human AGT has been reported to repair O\textsubscript{6}edGuo slowly in vitro (10). The defect in XP group A cells appears to be associated with the inability of DNA repair proteins to gain access to regions of chromatin containing DNA damage (11). This hypothesis would propose that NER proteins, and in particular the XPA factor, must open packed chromatin to allow AGT to repair O\textsubscript{6}edGuo lesions. It has been shown previously, however, that the XPA cell line used in these experiments is able to efficiently repair O\textsubscript{6}mdGuo, showing a greater than 80% reduction in this adduct within 24 h (12).

Another possible explanation for the dependence of O\textsubscript{6}edGuo repair on both repair pathways proposes that AGT participates directly in the removal of O\textsubscript{6}edGuo by the NER pathway. The removal of O\textsubscript{6}mGua and O\textsubscript{6}edGuo by NER has a precedent in bacterial photolyase (19), also recognizes and binds to UV-damaged DNA but is incapable of the direct photoreversal of lesions. Instead, XPE factor appears to act only as a damage recognition element to stimulate the removal of these lesions by NER (19).

The data for the repair of O\textsubscript{6}edThd and O\textsubscript{6}edThd stand in contrast to the O\textsubscript{6}edGuo results. Repair of O\textsubscript{6}edThd appeared to be minimal, with the half-lives of repair averaging in excess of 24 h (Fig. 1B). This is consistent with consensus views on the repair of O\textsubscript{4}-alkylthymine; reports on the repair of this adduct range from no repair at all to low but measurable repair (20). O\textsubscript{6}edThd appeared to be removed in a slow but consistent fashion, with half-lives of repair averaging around 20 h (Fig. 1C). This slow repair of O\textsubscript{6}edThd is also consistent with a published consensus on repair in mammalian cells (20). No correlation was seen between O\textsubscript{6}edThd repair and either AGT or NER.

In summary, these experiments have shown that human lymphoblasts require both AGT and NER pathways to efficiently repair O\textsubscript{6}edGuo adducts in genomic DNA. The repair rates of O\textsubscript{6}edThd and O\textsubscript{6}edThd are slow and do not appear to be related to these two DNA repair pathways.

### Acknowledgments

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### Table 1 Repair of O\textsubscript{6}edGuo in human lymphoblasts

<table>
<thead>
<tr>
<th>Exposure group</th>
<th>N cells</th>
<th>N with BrGua</th>
<th>X cells</th>
<th>X with BrGua</th>
<th>A cells</th>
<th>A with BrGua</th>
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<td>NER activity</td>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AGT activity</td>
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<td>+</td>
<td>+</td>
<td>-</td>
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</tr>
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<td>O\textsubscript{6}edGuo Half-life (h)</td>
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<td>53.0</td>
<td>43.4</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

*Fig. 2. A, diagram of two pyrimidine dimer repair pathways. E. coli and S. cerevisiae photolyase proteins bind dimers and can directly repair DNA (left pathway) or may act as damage recognition elements to stimulate NER (right pathway). Human XPE factor stimulates the removal of UV photoproducts by the pathway on the right. B, proposed model for repair of O\textsubscript{6}edGuo in human cells. AGT recognizes and binds to DNA containing either O\textsubscript{6}mdGua or O\textsubscript{6}edGuo. O\textsubscript{6}edGuo is subject to rapid direct repair by AGT (left pathway). Slow removal of ethyl groups from O\textsubscript{6}edGuo by AGT results in recruitment of NER proteins and excision of the damaged DNA (right pathway).*
References


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