Determinants of \(O^6\)-Alkylguanine-DNA Alkyltransferase Activity in Human Colon Cancer

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ABSTRACT

The DNA repair protein \(O^6\)-alkylguanine-DNA alkyltransferase (alkyltransferase) repairs cytotoxic DNA \(O^6\)-alkylguanine adducts induced by the nitrosoureas, triazines, and tetrazines. In this study, we determined whether there was a relationship between alkyltransferase activity in colon cancer and that of adjacent normal mucosa, and whether there were demographic patient characteristics which correlated with alkyltransferase expression in either tissue. Alkyltransferase activity and expression of the alkyltransferase gene, MGMT, were measured in 49 paired primary colon cancer samples and adjacent normal appearing mucosa. Alkyltransferase activity was found in all samples. The mean activity was higher in the tumor than the mucosa (\(r = 0.374, P < 0.01\)), although the low correlation coefficient suggested that multiple factors influence the alkyltransferase activity. MGMT mRNA could also be detected in all samples and was highly correlated with alkyltransferase activity (\(r = 0.64, P < 0.001\)). No correlation was found between alkyltransferase activity and age, or gender of the patient, or location of the tumor, although activity tended to be higher in patients with lower stage disease. Thus, alkyltransferase activity is present in most, if not all, colon cancer samples, suggesting that it could play an important role in chemotherapeutic resistance of human colon cancer. Patients with colon cancer would appear to be prime candidates for studies utilizing \(O^6\)-benzylguanine to deplete alkyltransferase prior to therapy with a nitrosourea, triazine, or tetrazine.

INTRODUCTION

With the introduction of \(O^6\)-benzylguanine (1) as a therapeutic biochemical modulator of nitrosourea resistance due to overexpression of the DNA repair protein \(O^6\)-alkylguanine-DNA alkyltransferase, there is renewed interest in evaluating DNA repair activity in human cancers. The alkyltransferase plays a pivotal role in drug resistance because it repairs DNA alkyl adducts at the \(O^6\) position of guanine (2), the site of the major cellular cytotoxic lesions induced by the nitrosoureas, triazines, and the tetrazines (3, 4). The alkyltransferase protein removes DNA adducts at this site by serving as the acceptor protein for the alkyl group in an irreversible transfer reaction (2). In the process of repair, the protein becomes inactivated. If a monoadduct, \(O^6\)-alkylguanine, is present, repair results in restoration of a normal guanine (5). On the other hand, exposure to chloroethylnitrosoureas results in a \(O^6\)-chloroethylguanine adduct which, if unrepaird, undergoes spontaneous rearrangement to an \(O^6\)-N'-ethanoguanine cyclic adduct prior to rearrangement to a very cytotoxic interstrand cross-link (6, 7). In these instances, alkyltransferase-mediated repair of the monoaduct restores the DNA to normal while repair of the \(O^6\)-N'-ethanoguanine adduct results in an alkyltransferase-DNA cross-link which appears to be less cytotoxic than the interstrand cross-link. The latter cannot be repaired by the alkyltransferase. Thus, the number of cytotoxic adducts removed by this process is equivalent to the number of alkyltransferase protein molecules in the cell, creating a threshold in cell resistance to DNA damage.

In human tumor samples, previous studies have shown a wide range of alkyltransferase activity in gastric tumors (8), melanomas (9), gliomas (10–12), and colon cancer (13, 14). Controversy remains over the absolute range in activity between individuals and between tumors, whether there are tumors with uniformly low alkyltransferase activity that should be susceptible to nitrosoureas, what proportion of tumors lack alkyltransferase activity and carry the "mer" phenotype (15), and whether there are demographic factors such as age or gender that define populations with either high or low alkyltransferase activity. For instance, Schold et al. (16) found that 5 (33%) of 15 brain tumor lines lacked detectable alkyltransferase activity and were sensitive to procarbazine in the xenograft model. Likewise, Silber et al. (11), Frosina et al. (10), Citron et al. (17), and Chen et al. (14) found low or undetectable alkyltransferase activity in approximately 22% of brain tumors. In other freshly isolated human lymphomas and melanomas as well as tumors from the breast, stomach, lung, and kidney, 5–12% were found to lack detectable alkyltransferase activity (8, 14, 17).

A number of groups have evaluated alkyltransferase in colon cancer samples in detail. Redmond et al. (13), Margison et al. (18), and our own group (19) found a 9–12-fold range of activity and a higher mean activity in colon tumors than in normal mucosa. Citron et al. (20) noted that 3 of 16 cancers had

1 The abbreviations used are: alkyltransferase, \(O^6\)-alkylguanine-DNA alkyltransferase; MGMT, \(O^6\)-methylguanine-DNA methyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
no detectable alkyltransferase activity and that activity was lower in tumors from males than females. While we have observed colon cancer cell lines lacking alkyltransferase activity, our initial study did not observe tumors that could be classified as mer− (19).

The striking range between individuals in alkyltransferase activity in the same tissue or tumor type remains unexplained (21). These differences may be due to differential expression of the alkyltransferase gene, MGMT (22–24), differences in stability of the alkyltransferase protein (25) or MGMT mRNA, heterogeneity in activity between histological types (11), differences in methodology between laboratories in the measurement of activity, and perhaps different rates of inactivation of the protein caused by repair of endogenous methylation of DNA at the O6 position of guanine (26). For these reasons, we analyzed 49 human colon tumors to define the correlation between alkyltransferase activity and MGMT mRNA levels in the tumors and adjacent mucosa, and determined whether there was a correlation between alkyltransferase activity and patient age, gender, tumor stage, or location.

MATERIALS AND METHODS

Colon Cancer Sample Collection. Patients with primary colon cancer underwent resection of the primary lesion and adjacent mucosa. The sample was immediately placed at 4°C in lactated Ringer’s solution and multiple small samples of both tumor and adjacent normal-appearing mucosa, which had been stripped from the submucosa, were resected and flash frozen and stored in the vapor phase of liquid nitrogen. Uniformity of processing was maintained by the Case Western Reserve School of Medicine National Cancer Institute-designated Cancer Center Tissue Procurement Facility under protocols approved by the University Hospitals of Cleveland Institutional Review Board. All sample sections had histological confirmation of the presence of tumor or normal mucosa. Samples with fibrosis, necrosis, inflammatory cell infiltrates, absence of the appropriate tissue (tumor or mucosa), or contamination by the complementary tissue, blood vessels, or lymphoidal tissue were excluded from further analysis. None of the selected samples had evidence of RNA degradation, an indication that these processing conditions were adequate to ensure accurate measurements of both protein activity and MGMT mRNA levels. Of 68 paired tumor and mucosal samples screened, 49 paired samples met the histological criteria for inclusion in the study.

Alkyltransferase Activity. The alkyltransferase assay has been previously described (21, 20). Briefly, 200 mg tissue sections were thawed directly into 500 μl cell extract buffer at 4°C as described (21). Tissues were then homogenized and sonicated. After centrifugation at 15,000 × g for 2 min to remove debris, activity was measured in whole tissue extracts by measuring the amount of [3H]methyl group removed from O6-[3H]methylguanine present in calf thymus DNA alkylated with N-[3H]methyl-N-nitrosourea. The alkylated [3H-methyl]O6-methylguanine and [3H-methyl]N7-methylguanine bases were separated by HPLC and quantified by liquid scintillation counting. Alkyltransferase activity was expressed as fmol O6-methylguanine removed/μg DNA or fmol O6-methylguanine removed/mg protein. Results are the mean of triplicate determinations for each sample.

Quantitation of MGMT mRNA Levels. Frozen samples were dropped into guanidium thiocyanate and homogenized. Total cellular RNA was isolated by cesium chloride centrifugation as described (27). Twenty μg total cellular RNA was separated by formaldehyde-agarose gel electrophoresis and the presence of MGMT mRNA was quantified by Northern blot analysis and internally standardized by blotting with GAPDH using methods we have previously described (27, 28). [32P]dCTP labeled human MGMT and human GAPDH cDNA probes were used. Hybridization bands for MGMT and GAPDH were quantified using a Bio-Rad laser scanning densitometer and compared to internal standards. All gels analyzed had band intensities within the linear range of the densitometer. Data are reported as the normalized absorbance of the hybridization signal for MGMT mRNA relative to GAPDH mRNA. The relative values of MGMT/GAPDH band intensity of tumor and mucosa from the same individual were then calculated and reported as the ratio between the two.

Statistical Correlations. The age and sex of each patient and the stage and location of each tumor sample was correlated, using linear regression analysis, with the alkyltransferase activity and the relative MGMT mRNA expression of each sample. Specific measurements were collected as noted above and mean values were used for each sample. A two-sided paired Student’s t test of significance was used with P < 0.05 considered significant.

RESULTS

Alkyltransferase Activity in Colon Cancer. Among the 49 patients in the study, there were 25 females (age, 31–88 years) and 24 males (age, 41–83 years). Four patients had villous adenoma, 5 Duke’s stage A, 14 Duke’s stage B, 15 Duke’s stage C, and 11 Duke’s stage D disease. Five patients had primary disease of the cecum, 24 had tumors of the right (ascending) colon, 2 had cancer of the transverse colon, 6 of the left (descending) colon, 7 of the sigmoid colon, and 5 of the rectum. Of the malignant tumors, 39 were moderately well differentiated, 5 were poorly differentiated, and 2 contained areas of both poorly and moderately well-differentiated cells.

Alkyltransferase activity could be measured in all normal-appearing mucosa and tumor samples. The level of activity ranged from 0.90–11.1 fmol/μg DNA in the normal mucosa and from 1.5–24.5 fmol/μg DNA in the tumor samples. Thus, none of the 49 tumor samples could be classified as mer−. The mean activity was significantly higher in the tumors than in the normal mucosa (9.1 ± 5.3 versus 7.4 ± 2.4 fmol/μg DNA, P < 0.05). However, the distribution of activity in both tumor and adjacent mucosa was not affected by age or gender. There was a significant correlation between mucosal and tumor alkyltransferase (Fig. 1), although the correlation coefficient was weak (r = 0.374, P < 0.01), indicating a multifactorial relationship. In a subset of samples, multiple sections of tumor or mucosa were sampled for alkyltransferase activity. As previously noted by Redmond et al. (13), similar protein activity was noted (variation, <25%) in multiple sections, indicating that inrasample
correlation (P < 0.01, r = 0.374) between the two.

variation was much less than intersample variation (data not shown).

To assess factors affecting the distribution of alkyltransferase activity in human colon cancer samples, the correlation between activity and tumor stage (Fig. 2A) and primary anatomical site (Fig. 2B) are shown. The mean level of activity was higher in the 4 cases of villous adenoma, 12.3 ± 8.3 fmol/μg DNA, and the 5 cases of Dukes' stage A carcinomas, 12.5 ± 5.3 fmol/μg DNA, than the mean levels in Dukes' stage B tumors, 7.6 ± 4.2 fmol/μg DNA, and Dukes' stage D tumors, 7.1 ± 3.8 fmol/μg DNA (P < 0.05), but was not significantly different from the Dukes' stage C tumors, 10.2 ± 5.1 fmol/μg DNA. On the other hand, there was no statistically significant correlation between alkyltransferase activity and anatomical site.

Laboratories differ in their reporting of alkyltransferase activity. We first proposed that it was appropriate to report activity on the basis of cellular DNA content because tissues vary widely in their relative levels of protein and DNA per cell (21). Since the alkyltransferase protein functions as a stoichiometric DNA repair protein, the cellular protection from DNA damage can be better estimated by reporting the activity based on DNA rather than protein content. Nonetheless, to allow comparison of our data to that of other laboratories that report activity on the basis of tissue protein content, Fig. 3 shows the very high degree of correlation between the alkyltransferase activity measured relative to DNA content with that relative to protein content. The conversion factor (slope of the linear regression line) was 33.1 μg protein/μg DNA for the tumor samples (r = 0.95) and 26.4 μg protein/μg DNA for the mucosal samples (r = 0.81). The high degree of correlation between the two values, particularly in the tumor samples, indicates minimal heterogeneity between the samples in the ratio of DNA:protein, a further confirmation of the histological finding that the selected samples consisted of tumor or normal mucosa rather than fibrosis, inflammation, or necrotic debris.

MGMT mRNA Levels in Colon Cancer. To assess the heterogeneity in MGMT mRNA levels in colon tumor samples, total cellular RNA was isolated from frozen sections of the same samples studied above and probed by Northern blot for hybridization to human MGMT cDNA and to the cDNA for GAPDH as a control. Fig. 4 shows a representative Northern blot of a group of paired tumor and mucosal samples from the same patients. Each Northern blot was analyzed by densitometry and the relative absorbance intensity of the MGMT mRNA band (standardized to GAPDH mRNA) from tumor samples was plotted against the alkyltransferase activity of each sample as shown in Fig. 5. There was a statistically significant relationship between the relative amount of MGMT mRNA in each sample and alkyltransferase activity (r = 0.64, P = 0.0013), although there was a sufficiently wide range to preclude using the MGMT mRNA to predict the alkyltransferase activity in an individual sample.

We then determined whether there was a correlation within individuals between the relative MGMT mRNA levels and alkyltransferase activity among tumor and mucosal samples. This would give a measurement of the concordance of expression between the normal and malignant tissues. The determination was made by plotting the ratio of alkyltransferase activity for tumor versus mucosa in each patient against the ratio of MGMT mRNA:GAPDH mRNA for tumor versus mucosa in the same patient. Fig. 6 shows the high correlation between the ratio of tumor:mucosa alkyltransferase activity and the ratio of tumor: mucosa MGMT mRNA for paired patient samples (r = 0.734, P < 0.0001). As observed with alkyltransferase activity, no statistically significant correlations were found between MGMT mRNA levels and the demographic factors (data not shown).

DISCUSSION

This study found that all 49 human colon tumor samples had detectable levels of alkyltransferase activity and that tumors had a somewhat higher mean level of alkyltransferase activity than paired, adjacent normal-appearing mucosa. In addition, there was a significant correlation between tumor and mucosal values from the same individual, although there was a wide degree of scatter in the observed values and a correlation coefficient of only r = 0.374. This suggests that there are many other factors contributing to alkyltransferase expression in colon tumors which can only partially be explained by the level of activity in the normal-appearing adjacent mucosa.

Nonetheless, unlike gliomas (10–12), melanomas (9), gastric tumors (8), or esophageal tumors (14), the vast majority of human colon cancers express alkyltransferase. Based on in vitro and xenograft data (29–31), expression of the alkyltransferase appears to provide a major source of enzymatic resistance to nitrosoureas and related compounds. Over 90% of colon tumor samples had alkyltransferase activity of greater than 1.0 fmol/μg DNA (or approximately 33 fmol/mg protein), which, in colon cancer cell lines, has been strongly associated with nitrosourea resistance both in vitro and in vivo (19, 32). We propose that this is a major mechanism of resistance to nitrosoureas and triazines observed in patients with colon cancer (33). Conversely, the presence of high alkyltransferase in colon cancer makes it a good tumor in which to evaluate biochemical modulation of the
alkyltransferase using the inhibitor O\textsuperscript{6}-benzylguanine. When combined with 1,3-bis(2-chloroethyl)-1-nitrosourea, O\textsuperscript{6}-benzylguanine has been highly efficacious in colon cancer xenografts resistant to 1,3-bis(2-chloroethyl)-1-nitrosourea alone because it is able to completely deplete alkyltransferase activity in the tumor with tolerable toxicity to normal host tissues (34).

As in other human cells and tissues, we observed a wide range of alkyltransferase activity within both colon tumors (range, 1.5–24.5 fmol/\(\mu\)g DNA) and colon mucosa (range, 0.90–11.1 fmol/\(\mu\)g DNA). The lowest level of activity observed in colon tumors was greater than the lowest activity reported with other tumors (see above). On the other hand, the maximum activity (reported based either on activity per \(\mu\)g DNA or per mg protein) was similar to the maximum levels of activity noted in gastric tumors (8), meningiomas (10), and ovarian cancer (14) but higher than that reported for a number of other tumor types (11). Whether high alkyltransferase confers a selective advantage to mucosal or tumor cells remains speculative. Transgenic mice overexpressing alkyltransferase in a variety to tissues are protected from carcinogens but have a normal growth, development, and aging pattern (28). Perhaps the colonic mucosa is exposed to a variety of carcinogens and toxins which alkylate at the O\textsuperscript{6} position of guanine and cells expressing high levels are protected from the cytotoxic effects of these chemicals (35). Alternatively, perhaps MGMT is one of a number of genes whose expression is up-regulated during colon tumor progression. In this regard, it is of interest that the alkyltransferase activity was higher in villous adenomas and Dukes' stage A carcinomas than in more advanced tumors. While this might indicate that up-regulation of MGMT expression occurs early during the carcinogenic process, it could also be confounded by differing amounts of connective tissue and stromal cells present in the tumor samples. In recent studies, we found that these regions contain lower levels of alkyltransferase than the glandular regions of the tumor (36).

MGMT mRNA measurements have been proposed as an alternative to protein activity to predict DNA repair capacity or nitrosourea resistance within an individual tumor sample. Indeed, we observed a high degree of correlation between the level of alkyltransferase protein and MGMT mRNA in paired colon tumors and mucosal samples, raising the possibility that MGMT mRNA measurement might be a useful screening test for human tumors. Furthermore, these data suggest that within colonic tissues, control of alkyltransferase activity occurs predominantly

![Fig. 2](image-url)  
*Fig. 2* Distribution of alkyltransferase activity in colon tumors. *A*, distribution of alkyltransferase activity relative to the tumor stage. VA, villous adenoma; A-D, Dukes' stages. *B*, distribution of alkyltransferase activity relative to the tumor location. C, cecum; R, right (ascending) colon; T, transverse colon; L, left (descending) colon; S, sigmoid colon; E, rectal tumors.

![Fig. 3](image-url)  
*Fig. 3* Correlation between alkyltransferase activity based on the protein and DNA concentrations in colon mucosa and tumor samples. For each sample, the alkyltransferase activity is plotted per mg protein and per \(\mu\)g DNA. The slope for tumors (*solid line*) was 33.1 fmol alkyltransferase/mg protein/\(\mu\)g DNA \((r = 0.95)\) and for mucosa (*dashed line*) was 26.4 fmol alkyltransferase/mg protein/\(\mu\)g DNA \((r = 0.81)\).
Fig. 4 Northern blot analysis of MGMT expression in paired colon samples. Total RNA was prepared from the same paired tumor and normal mucosal samples assayed for alkyltransferase activity. Following electrophoresis and transfer to membranes, blots were hybridized sequentially with $^{32}$P-labeled MGMT and GAPDH cDNA. The bands signifying specific hybridization with the appropriate probe are indicated. $N$, normal-appearing mucosa; $T$, tumor. A representative blot is shown, with 6 pairs of samples.

Fig. 5 Correlation between tumor alkyltransferase activity and MGMT expression. The relative expression of MGMT was normalized to GAPDH expression and normalized between blots and expressed as the ratio of the two. This was plotted against the alkyltransferase activity of each sample. The relationship was significant, $r = 0.64$, $P = 0.0013$.

Fig. 6 Correlation between the ratio of tumor:mucosa alkyltransferase activity and the ratio of tumor:mucosa MGMT expression. As in Fig. 5, the relative expression of MGMT in mucosa samples was normalized to GAPDH expression. In this figure, the ratio of MGMT:GAPDH expression is compared to the alkyltransferase activity by comparing the ratio between tumor and mucosa. This was plotted against the ratio of alkyltransferase activity, tumor versus mucosa. In all instances, paired samples from the same individual are shown. The relationship was significant, $r = 0.73$, $P < 0.0001$.

at the level of transcription and that posttranscriptional modification and differences in alkyltransferase protein stability play little if any role. In addition, this process appears to be reasonably constant within and between individuals. This is an important issue because endogenous methylation in the gut and endogenous proteases have been proposed as possible components of intracellular alkyltransferase inactivation systems (24, 37). If these factors functioned differentially between patients, less correlation would have been observed. Thus, our data might be extrapolated to indicate: (a) the existence of a common process regulating alkyltransferase activity once the level of gene expression has been determined and (b) that endogenous methylation is an unlikely modulator of alkyltransferase activity in vivo.

A number of laboratories are evaluating factors affecting MGMT gene expression. In most instances, the identified factors determine whether or not the gene is expressed and not the relative level of expression. For instance, methylation of the promotor prevents expression (38) whereas methylation of the
body of the gene is permissive to expression (23). In addition, Harris et al. (24) suggest that transcription factors may modulate MGMT gene expression. We addressed whether demographic factors influenced the relative level of alkyltransferase activity. There was no significant correlation between the tumor location, the age or gender of the patient, and either alkyltransferase activity or MGMT mRNA levels. These findings suggest that alkyltransferase activity may not be genetically determined or influenced by aging. Also, it is unlikely that oncogenic processes which occur at various stages in colon carcinogenesis and tumor progression, such as K-ras, p53 mutations, or mdm-2 overexpression, affect MGMT expression in a predictable fashion.

Human colon cancer expresses the MGMT gene and the alkyltransferase protein at sufficient levels to explain its observed clinical and preclinical resistance to nitrosoureas. The correlation between MGMT mRNA and alkyltransferase activity suggests that individuals share a common posttranscriptional control of the alkyltransferase level in colonic cells. Biochemical modulation of the alkyltransferase protein in colon tumors and/or inhibition of MGMT mRNA transcription would appear to be fruitful targets for clinical trials sensitizing colon tumors to nitrosoureas and related triazines and tetrazines.

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