Increased Sensitivity of Transforming Growth Factor (TGF) β1 Null Cells to Alkylation Agents Reveals a Novel Link between TGFβ Signaling and O6-Methylguanine Methyltransferase Promoter Hypermethylation*

Hisaharu Yamada, Kinnimulki Vijayachandra, Carrie Penner, and Adam Glick†‡§

From the †Toxicology Laboratory, Pharmaceutical Research Laboratories, Taisho Pharmaceuticals, Tokyo 170-8633, Japan and ‡Laboratory of Cellular Carcinogenesis and Tumor Promotion, NCI, National Institutes of Health, Bethesda, Maryland, 20892

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Inactivation of the transforming growth factor β (TGFβ)-signaling pathway and gene silencing through hypermethylation of promoter CpG islands are two frequent alterations in human and experimental cancers. Here we report that nonneoplastic TGFβ1−/− keratinocyte cell lines exhibit increased sensitivity to cell killing by alkylating agents, and this is due to lack of expression of the DNA repair enzyme O6-methylguanine DNA methyltransferase (MGMT). In TGFβ1−/− but not TGFβ1+/− cell lines, the CpG dinucleotides in the MGMT promoter are hypermethylated, as measured by restriction enzyme analysis and methylation specific polymerase chain reaction. In one unstable TGFβ1−/− cell line, loss of the wild type TGFβ1 allele correlates with the appearance of methylation in the MGMT promoter. Bisulfite sequencing shows that in the KO3 TGFβ1−/− cell line nearly all of the 28 CpG sites in the MGMT promoter 475 base pairs upstream of the start site of transcription are methylated, whereas most are unmethylated in the HI TGFβ1+/− line. Treatment of the TGFβ1−/− cell lines with 5-azacytidine causes reexpression of MGMT mRNA and demethylation of CpG islands in the promoter. Analysis of the time course of methyltransferase using methylation-specific polymerase chain reaction shows a lack of methylation in primary TGFβ1−/− keratinocytes and increasing methylation with passage number of immortalized clones. Subcloning of early passage clones reveals a remarkable heterogeneity and instability of the methylation state in the TGFβ1−/− keratinocytes. Thus, the TGFβ1−/− genotype does not directly regulate MGMT methylation but predisposes cells to immortalization-associated MGMT hypermethylation.

Inactivation of tumor suppressor genes is a common feature of cancer development in humans and animal models. There is increasing evidence that methylation of normally unmethylated CpG islands in gene promoters is an important epigenetic mechanism for transcriptional inactivation of tumor suppressor and DNA repair genes (1–3). One DNA repair gene that is frequently hypermethylated in tumors is methylguanine methyltransferase (MGMT)1 (4, 5). MGMT removes alkyl adducts from O6-guanine residues by transferring the alkyl group to an active cysteine residue within its sequence in a reaction that inactivates further enzymatic activity (6). Since O6-alkylated guanine can mispair with thymine during replication to cause transversions as well as cross-link with cytosines on the opposite DNA strand (6), cells that are deficient in MGMT activity may be more susceptible to mutation and, hence, cancer development or progression. Supporting this role in cancer development, transgenic animals overexpressing MGMT are resistant to tumor formation induced by alkylating agents (7), whereas MGMT null animals exhibit an increased frequency of methylnitrosourea-induced tumors (8).

TGFβ1 is a member of a large family of multifunctional secreted polypeptides that are potent growth inhibitors of epithelial cells (9). In human cancers and animal models of multistage carcinogenesis, inactivation of TGFβ signaling through mutations in the receptors (10–12) or intracellular Smad proteins (13–16) is associated with accelerated premalignant progression and malignant conversion. In the mouse epidermal carcinogenesis model, TGFβ1 acts as a tumor suppressor since progression of chemically induced benign tumors is associated with loss of TGFβ1 (17, 18), and genetic inactivation of the signaling pathway in keratinocytes leads to rapid progression to squamous cell carcinoma (19, 20). To understand the mechanism by which loss of autocrine TGFβ signaling leads to accelerated tumor progression, we established a series of non-neoplastic, spontaneously immortal cell lines derived from newborn mouse TGFβ1+/− and −/− keratinocytes. The TGFβ1−/− cell lines had a significantly higher level of gene amplification than controls (21). To explore further the role of TGFβ1 in genomic stability, we have examined the response of TGFβ1+/− and TGFβ1−/− cell lines to different DNA-damaging agents. Our results show that the TGFβ1−/− cell lines are specifically more sensitive to cell killing by alkylating agents, and this is due to a lack of expression of MGMT mRNA and enzyme. Southern blot, MSP, and bisulfite sequencing of the MGMT promoter indicates that the lack of expression is due to hypermethylation of CpG islands in the MGMT promoter. This is the first demonstration of a link between TGFβ1 expression and aberrant promoter methylation, and it could have important implications for mechanisms of tumor progression caused by inactivation of TGFβ signaling.

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†To whom correspondence should be addressed: Laboratory of Cellular Carcinogenesis and Tumor Promotion, Bldg. 37 3B19 National Cancer Institute, NIH, Bethesda, MD 20892. Tel.: 301-496-3248; Fax: 301-496-8709; E-mail: glicka@dc37a.nci.nih.gov

1 The abbreviations used are: MGMT, O6-methylguanine DNA methyltransferase; PCR, polymerase chain reaction; MSP, methylation-specific PCR; TGF, transforming growth factor.

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EXPERIMENTAL PROCEDURES

Cell Culture—The TGFβ1+/− and TGFβ1−/− cells are spontaneously immortal, clonally derived non-tumorigenic keratinocyte cell lines isolated from primary epidermal cultures of newborn mice from the TGFβ1+/− strain (19, 22). Cells were routinely cultured in Earle’s minimum essential medium, 8% chexeled fetal calf serum, 0.05 mM CaCl2, and antibiotics. Unless indicated, all cell lines were used between passages 20–50. Balb/c keratinocytes were isolated from newborn Balb/c mice using standard techniques (23) and cultured for 3–5 days before isolation of DNA. For 5-azacytidine treatment, cells were seeded at 1 × 105 cells/175-cm² tissue culture flask, and exponentially growing cells were treated with 1 μM 5-azacytidine (Sigma) for 48 h. After a 3-day recovery period, the treatment protocol was repeated, after which DNA and RNA were isolated. To isolate subclones of the KO3 passage 8, cells were plated at low density in 175-cm² tissue culture dishes, and colonies that grew out were ring-cloned, expanded to a T-75 flask, and DNA-isolated. To generate continuous lines, primary TGFβ1−/− keratinocytes were cultured in medium containing 10 ng/ml keratinocyte growth factor for several weeks until immortal colonies grew out. These colonies were pooled and passage twice before seeding at low density and ring-cloning. The TGFβ1 wild type keratinocyte cell line, NHK4, was clonally derived from newborn keratinocyte cultures isolated from p53−/− mice (24). The B8 and M3 TGFβ1 wild type cell lines were derived from newborn epidermis from control mice of the c-fos−/− line (25).

Clonogenic Survival Assay—Approximately 500 cells of each cell line were seeded into 60-cm² culture dishes and allowed to attach for 24 h. Cells were treated with serial dilutions of the different drugs for 1 h in complete medium placed in 20-cm² dishes. Balb/c keratinocytes were isolated from newborn keratinocyte cultures isolated from p53−/− mice (24). The B8 and M3 TGFβ1 wild type cell lines were derived from newborn epidermis from control mice of the c-fos−/− line (25).

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Methylation-specific PCR—Methylation-specific PCR was used to identify primers specific for either the unmethylated or methylated modified DNA (31). 1 μg of genomic DNA was modified with sodium bisulfite and purified (31). For the mouse MGMT promoter, the following primer pairs were used: M1-5′-GGATGTTTCTGTCATGATTTTG-3′ and M2-5′-TGGTCTTACGTGGGCTCTC-3′, and M3-5′-GGATGTTTCTGTCATGATTTTG-3′ and M4-5′-TGGTCTTACGTGGGCTCTC-3′. PCR reactions were carried out in a 20-μl volume using 100 ng of modified DNA, 1× PCR buffer (PerkinElmer Life Sciences), 0.2 units of AmpliTaq Gold (PerkinElmer Life Sciences) under the following amplification conditions: 94 °C for 90 s, 60 °C for 90 s, 72 °C for 2 min for 35 cycles. With these conditions, no amplification was observed with the wild type primers on modified DNA or with unmethylated/methylated primers on unmethylated DNA. Controls without DNA were performed with each set of PCR. PCR products were electrophoresed through a 2% agarose gel, and visualized by ethidium bromide fluorescence. The PCR fragment was cloned into pCR2.1 using the TA-cloning method (Invitrogen), and selected clones were sequenced with M13 primers using the dye terminator DNA-sequencing kit (PE Applied Biosystems) with a PerkinElmer ABI Prism 377 DNA sequencer.

RESULTS

Sensitivity of TGFβ1−/− Keratinocytes to Alkylating Agents—TGFβ1+/− and −/− keratinocyte cell lines were treated with different DNA-damaging agents, and the ability of the treated cells to form viable colonies was used as a measure of relative DNA repair capacity. Fig. 1 shows that for UV and γ irradiation or cisplatin there was no consistent difference in the IC50 for inhibition of colony formation between the TGFβ1−/− and TGFβ1+/− genotypes. Similar dose-response curves were obtained with the topoisomerase inhibitors camptothecin or etoposide (data not shown). However, the TGFβ1−/− cell lines were 5-fold more sensitive to cell killing by the alkylating agent MNNG than the TGFβ1+/− lines. Similar results were obtained with methylmethotrexate (data not shown). TGFβ1+/− cell lines derived independently from other transgenic lines (B8, M3, NHK4) had sensitivities to MNNG that were similar to the TGFβ1+/− cell lines. Since the NHK4 cell line is p53−/− (24), the increased sensitivity to alkylating damage is specific to the TGFβ1−/− genotype.

Increased Sensitivity to MNNG Due to Absence of MGMT—MNNG and methylmethotrexate produce a high proportion of O6-methylguanine adducts that are specifically repaired by the enzyme O6-methylguanine DNA methyltransferase (MGMT) (6). Cells that lack this enzyme exhibit increased sensitivity to cell killing by alkylating agents (4). The level of MGMT enzyme activity in the TGFβ1+/− and TGFβ1−/− keratinocyte cell lines was determined using a well characterized assay that measures the ability of crude cellular extracts to transfer a [3H]methyl group from M. luteus DNA to protein (26). Table I shows that all TGFβ1+/−/cell lines had levels of MGMT activity ranging from 0.102–0.255 pmol/mg of protein. Treatment of these cells with O6-benzylguanine, a specific inhibitor of MGMT, eliminated enzyme activity. In contrast, 4/5 TGFβ1−/− keratinocyte cell lines had no detectable MGMT activity, whereas in all KO3 the levels were 0.255 pmol/mg of protein, barely above the background. Northern blot analysis showed that the lack of MGMT enzyme in the TGFβ1−/− cell lines was due to the absence of the MGMT transcript (Fig. 2). There was no difference between the two genotypes in expression of another repair enzyme, methylpurine glycosylase, which removes N-methylpurines and other damaged purines in DNA (33) (Fig. 2). No difference in hybridization pattern was seen when restriction-digested genomic DNA from the TGFβ1+/− and TGFβ1−/− cell lines was hybridized to a MGMT cDNA probe (data not shown). These results indicate...
that the lack of MGMT expression in the TGFβ1−/− cell lines was not due to deletion or rearrangement of the MGMT gene.

Hypermethylation of MGMT Promoter in TGFβ1−/− Keratinocytes—A frequent mechanism for inactivation of MGMT expression in human tumors and tumor cell lines is hypermethylation of CpG islands in the gene promoter (3, 34). Initial Southern blot analysis of methylation in the MGMT promoter revealed specific methylation of the −328 HpaII site in two TGFβ1−/− cell lines (not shown). To assay the methylation status of the MGMT promoter in all cell lines, we developed a methylation-specific PCR assay for the mouse MGMT promoter (31). Treatment of DNA with bisulfate converts unmethylated cytosines to uracil, whereas methylated cytosines are resistant to this chemical modification (31). Specific primers were generated to distinguish methylated (M) from unmethylated (U) DNA in the mouse MGMT promoter based on sequence alterations following bisulfite modification. Fig. 3 shows that in all of the TGFβ1+/− lines as well as Balb/c keratinocytes, a PCR product was obtained after bisulfite modification only with the unmethylated PCR primers and not with the methylated primers. Thus some or all of the CpG sites within these primer sequences are unmethylated in the control cells. In contrast, the methylated primer pair yielded a strong PCR product with bisulfite-modified DNA from all of the TGFβ1−/− lines, whereas variable levels of PCR product was generated with the unmethylated primer pair. To examine the extent of methylation in the MGMT promoter, DNA from the KO5 and H1 cell lines at passage 35 was subjected to bisulfite modification followed by sequencing of PCR products amplified with non-methylation-specific primers. Of 28 CpG dinucleotides between −475 and the start of transcription, virtually all were unmethylated in different PCR clones from the H1 cell line. In contrast, between 57 and 82% of the CpG dinucleotides

![Figure 1](http://example.com/fig1.jpg)  
**FIG. 1. TGFβ1−/− keratinocyte cell lines are more sensitive to cell killing by MNNG.** Shown is the colony-forming ability of TGFβ1+/+, +/−, and −/− keratinocyte cell lines after treatment with MNNG (A), UV (B) and γ irradiation, or cisplatin (D). Cells were plated at clonal density as described under “Experimental Procedures,” allowed to recover 24 h, and then treated with the indicated concentration of the drug or irradiated. Colonies, which formed 7–14 days after treatment, were stained and counted, and the number of colonies formed at each dose was determined as a percentage of the untreated control. Each dose was done in triplicate. MNNG treatment was repeated 4 times with identical results. TGFβ1 wild type lines were generated from the epidermis of p53−/− mice (NHK4) and from the wild type genotype of c-fos−/− mice (B8, M3).

![Figure 2](http://example.com/fig2.jpg)  
**FIG. 2. TGFβ1−/− cell lines do not express MGMT mRNA.** Northern blot analysis of MGMT expression in poly(A)− RNA isolated from TGFβ1+/+ and TGFβ1−/− cell lines is shown. The Northern filters were sequentially hybridized to a human MGMT cDNA probe (28), a methylpurine glycosylase (MPG) cDNA, and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. Exposure times were 3–4 days for MGMT and methylpurine glycosylase (MPG) and 1 h for glyceraldehyde-3-phosphate dehydrogenase.

| Genotype   | Cell line | MGMT activity |
|------------|-----------|---------------|
| TGFβ1−/−   | KO1       | Not detectable|
|            | KO2       | Not detectable|
|            | KO4       | Not detectable|
|            | KO5       | Not detectable|
|            | KO6       | 0.005 ± 0.001 |
| TGFβ1+/−   | H1        | 0.255 ± 0.012 |
|            | H4        | 0.217 ± 0.014 |
|            | H5        | 0.102 ± 0.011 |
|            | H1 + BZG  | 0.012         |
|            | H4 + BZG  | 0.007         |

![Table 1](http://example.com/table1.jpg)  
**Table 1**  
Absence of MGMT enzyme activity in TGFβ1−/− keratinocyte cell lines

MGMT enzyme activity (pmol of 3H removed/mg of protein) was measured in crude cellular extracts of the TGFβ1−/− and +/− keratinocyte cell lines as described under “Experimental Procedures.” Values represent the average of 2–3 independent determinations. MGMT activity was inhibited by O'-benzylguanine (BZG), indicating specificity of the enzyme activity measured for MGMT.
Methylation is an inherent property of the TGFβ1/−/− and TGFβ1+/− cell lines, and Balb/c keratinocytes (B) was modified with bisulfite as described under "Experimental Procedures" and analyzed for methylated CpG sites using PCR primers, which distinguish unmethylated (U) and methylated (M) sequences. Amplification did not occur with either primer set using unmethylated DNA. PCR products were analyzed on a 2% agarose gel and visualized by ethidium bromide fluorescence.

were methylated in different PCR products sequenced from the KO3 cell line (Fig. 4). Apart from a region just upstream from −25, which was free of methylation in all clones, virtually the entire promoter was methylated.

5-Azacytidine Causes Demethylation and Reexpression of MGMT mRNA—To further strengthen the link between methylation of the MGMT promoter and lack of mRNA expression in the TGFβ1/−/− lines, these cells were treated with 5-azacytidine to block methylation. Fig. 5A shows that after treatment of the TGFβ1/−/− cell lines with 5-azacytidine MGMT mRNA was reexpressed (Fig. 5A). This correlated with demethylation of the MGMT promoter as measured by an increase in the unmethylated- relative to the methylated-specific PCR product after bisulfite modification and MSP in KO1 KO3 and KO6 DNA (Fig. 5B).

Relationship of TGFβ1 to Methylation Is Indirect and Linked to Immortalization—To examine the correlation between the lack of autocrine TGFβ1 expression and aberrant methylation of the MGMT promoter, we analyzed the MGMT methylation status in the unstable TGFβ1+/− cell line H7. Previous studies show that the TGFβ1 wild type allele in this cell line is lost with increasing passage (21). Methylation-specific PCR analysis of the MGMT promoter in H7 showed that at passage 9 a PCR product was generated only with the unmethylated primer pair, but by passage 12, a methylated band is evident, and this increases relative to the unmethylated band by passage 24 (Fig. 6). These results suggest that there is a close association between autocrine TGFβ1 expression and hypermethylation of the MGMT promoter. However, short or long term treatment of cells with TGFβ1 did not alter MGMT expression. Table II shows that treatment of Balb/c primary keratinocytes with 0.5 ng/ml TGFβ1 for 48 h did not alter MGMT enzyme activity, nor did a similar treatment induce expression of MGMT in the KO1 and KO3 cell lines. Additionally, treatment of the KO3 cells with exogenous TGFβ1 at 50 pg/ml for up to 1 month did not reduce methylation of the MGMT promoter. These results suggest that the effect of a TGFβ1 null genotype on methylation of the MGMT promoter is indirect or that once established, the methylation pattern cannot be reversed by addition of TGFβ1. To test whether hypermethylation is an inherent property of the TGFβ1 null cells, we examined MGMT promoter methylation by MSP in primary keratinocytes of all genotypes as well as TGFβ1/−/− cells at different times of culture. Table II and Fig. 7A show that regardless of TGFβ1 genotype, all primary keratinocytes expressed high levels of MGMT enzyme and have an unmethylated MGMT promoter. However, with continued passage of the null cells in culture, a faint band was reproducibly detected with the methylated PCR primers in passage 8 TGFβ1/−/− cells, and this increased in proportion such that by passage 35 the predominant band was with the methylated-specific primers. These results suggest that the methylation state of the MGMT promoter is unstable in the KO keratinocytes. To test this more directly, we subcloned the null keratinocytes at passages 2 and 8 and examined methylation status of these clones by MSP. Fig. 7, B and C, shows that in subclones from both, there is considerable variability in the methylation state of the MGMT promoter. DNA from some clones was completely methylated (clones 1, 3, 4, 6, 8), whereas in others it was unmethylated (clones 2, 9, 10), and the remainder had amplification with both unmethylated- and methylated-specific PCR primers. Thus the methylation state of the MGMT promoter in the TGFβ1/−/− cells exhibits clonal- and time-dependent variability.

DISCUSSION

Inactivation of the TGFβ signaling pathway is a common event in the progression of human and experimental cancers. In the multistage skin carcinogenesis model v-ras retrovirus transduced TGFβ1/−/− primary keratinocytes undergo rapid aneuploidy in culture (35) and progress to squamous cell carcinoma, whereas control genotypes show limited instability and progression in vivo (19). Nontumorigenic cell lines derived from primary cultures of TGFβ1/−/− keratinocytes also have a high frequency of gene amplification N-(phosphonacetyl)-L-aspartate (21), suggesting that loss of autocrine TGFβ1 signaling results in decreased genomic stability and rapid malignant progression. Here we show that these TGFβ1/−/− cell lines also exhibit a specific defect in the DNA repair enzyme MGMT, which is crucial to repair of adducts caused by alkylating agents. Relative to the TGFβ1+/+ cell lines, all of the TGFβ1/−/− cell lines had a 5-fold increase in sensitivity to cell killing by MMNG, which produces O'-methylguanine adducts with high frequency. In addition, the TGFβ1/−/− cell lines did not have measurable MGMT enzyme activity or express MGMT mRNA, the enzyme responsible for repair of this DNA lesion (6). In contrast there was no significant difference in sensitivity to γ or UV irradiation, cisplatin, or topoisomerase inhibitors. All of these agents produce lesions that utilize repair pathways distinct from MGMT. There was also no difference in mRNA expression of another repair enzyme, methylpurine glycosylase, between the two TGFβ1 genotypes, pointing to a specific defect in MGMT expression. Using a combination of Southern blot analysis with methylation-sensitive restriction enzymes, methylation-specific PCR, and bisulfite sequencing, we found that the promoter region of the mouse MGMT gene was also heavily methylated in the MGMT-deficient TGFβ1/−/− cell lines but not in the MGMT proficient TGFβ1+/− cell lines. Furthermore, treatment of the TGFβ1/−/− lines with 5-azacytidine caused reexpression of MGMT mRNA expression and demethylation of the promoter. These results are in agreement with many studies of MGMT expression in human cancers and tumor cell lines that show a strong correlation between promoter methylation and silencing of MGMT expression (4, 5) and support the idea that hypermethylation of the MGMT promoter specifically in the TGFβ1/−/− cell lines is responsible for lack of expression.

Bisulfite sequencing revealed that although the MGMT promoter was hypermethylated in the KO3 cell line, there was heterogeneity between individual DNA clones sequenced and regions of both frequent and rare methylation. Similar heterogeneity of methylation at specific CpG sites between individual DNA clones was also found by bisulfite sequencing of the human MGMT promoter from the Mer- BE colon tumor cell line (34). Whether this reflects cellular or allelic variability is not clear, but it must reflect an inherent variability in the process of methylation itself. The regions from −450 to −100 and −25 to +25 were highly methylated in all KO3 DNA copies sequenced, whereas there was infrequent methylation in the region from −80 to −30. It is remarkable that a similar regional pattern of CpG methylation is found in the MGMT...
promoter of the BE and HeLa S3 tumor cell lines, with a region of rare methylation between −100 to −30 surrounded by highly methylated regions on either side (34) even though there is no sequence homology between the mouse (30) and human (36) MGMT promoters. 6/6 TGFβ1−/− cell lines and none of the TGFβ1+/− cell lines lacked MGMT expression and had a hypermethylated MGMT promoter. We found no increase in sensitivity to MNNG in a p53−/− cell line and did not have apparent methylation as judged by the MSP assay, but rather this analysis showed that methylation increased specifically during passaging of the TGFβ1−/− cells.

FIG. 4. Bisulfite sequencing reveals extensive methylation of the MGMT promoter in KO3 cells but not H1 cells. Bisulfite-modified genomic DNA was sequenced as described under “Experimental Procedures.” Lollipops represent individual CpG sites within the MGMT promoter. The methylation status of each site for individual PCR clones sequenced is indicated by an open box (unmethylated) or closed box (methylated). Four subcloned PCR products were sequenced for the H1 line, and seven were sequenced for the KO3 line. The arrow indicates the start site of transcription.

FIG. 5. Demethylation of the MGMT promoter with 5-azacytidine causes reexpression of MGMT mRNA. A, 5-azacytidine induces MGMT mRNA expression in TGFβ1−/− cell lines. Northern blot analysis of MGMT mRNA expression in TGFβ1−/− cell lines treated with 1 μM 5-azacytidine (5-AzaC) for 48 h. mRNA isolated from treated or untreated cells was hybridized to a human MGMT cDNA probe and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Exposure time was 1 week for MGMT. B, reduced MGMT promoter methylation in 5-azacytidine-treated TGFβ1−/− cell lines. Genomic DNA from control and treated cells was modified with bisulfite as described under “Experimental Procedures” and subjected to methylation-specific PCR using primers specific for unmethylated (U) and methylated (M) sequences.

FIG. 6. Loss of wild type TGFβ1 allele in heterozygote cell line correlates with increased methylation of the MGMT promoter. Methylation-specific PCR analysis of H7 cell line at different passages. The MGMT promoter is initially unmethylated in P9 of this cell line, correlating wild type TGFβ1 allele. In DNA of P12 and P24 cells there is an increase in MGMT promoter methylation corresponding to loss of the wild type allele. U, unmethylated-specific primer; M, methylated-specific primer.

primary TGFβ1−/− null keratinocytes were MGMT-proficient and did not have apparent methylation as judged by the MSP assay, but rather this analysis showed that methylation increased specifically during passaging of the TGFβ1−/− cells.

FIG. 7. MGMT methylation increases with passage in TGFβ1−/− cells and exhibits clonal variation. A, methylation of the MGMT promoter is not detected in primary cultures of TGFβ1−/− keratinocytes but increases with passage. MSP analysis of MGMT promoter methylation in primary cultures of TGFβ1−/− (1) and TGFβ11+1− keratinocytes (2), passage 2 mass cultures of immortalized TGFβ1−/− keratinocytes (3), passage 8 culture of KO3 cell line (4), passage 32 of KO3 cell line (5), and passage 50 of KO3 cell line (6). B, variable methylation in clones of passage 2 TGFβ1−/− keratinocytes. Colonies of keratinocytes were ring-cloned and expanded, and the isolated DNA was subjected to bisulfite modification and MSP. C, variable methylation in clones of passage 8 KO3 cell line. Colonies of KO3 were ring-cloned and expanded, and the isolated DNA was subjected to bisulfite modification and MSP. U, unmethylated-specific primer; M, methylated-specific primer.

TABLE II

| Cell type       | Treatment | MGMT activity |
|-----------------|-----------|---------------|
| Primary keratinocytes | TGFβ1 0.5 ng/ml | 0.205 ± 0.014 |
| Balb/c          | None      | 0.218 ± 0.009 |
| TGFβ1+/+        | None      | 0.162 ± 0.003 |
| TGFβ1+/−        | None      | 0.223 ± 0.002 |
| TGFβ1−/−        | None      | 0.213 ± 0.002 |
| Cell line       |           |               |
| KO1             | TGFβ1 1.0 ng/ml | Not detected |
| KO3             | TGFβ1 1.0 ng/ml | Not detected |
Whether this represents a specific growth advantage of rare cells with MGMT methylation and TGFβ-signaling defects or ongoing methylation of the promoter in the absence of TGFβ signaling will require bisulfite sequencing of cells at different passage number for clarification. Although methylation was undetectable in passage 2 and slightly detectable in passage 8 TGFβ1/−/− cells, subclones of these passages exhibited variable methylation ranging from none to complete. Since the MSP assay is reported to detect methylated alleles at a frequency of 0.1% (31), it seems unlikely that the appearance of methylated alleles with such high frequency in the subclones represents enrichment of rare cells with methylation. Previous studies with fibroblasts show that genes unmethylated in adult tissues or primary cell cultures become methylated in immortal cell lines that grow out after crisis (37). A plausible hypothesis is that TGFβ1/−/− cells are more susceptible to hypermethylation, and this occurs randomly at the MGMT locus with every cell division. Such a model would allow for recovery of clones containing MGMT alleles that were unmethylated, completely methylation, or both.

An important question left unresolved is how loss of autocrine TGFβ1 could be linked to hypermethylation. TGFβ1 could directly regulate expression of one of the DNA methyltransferases (DNMT1–3), a demethylase (38), or proteins that bind to methylated DNA (39, 40). Since DNMT1 is both regulated by Rb (41) and forms a complex with Rb, EZF1, and HDAC1 (42), it is possible that altered TGFβ1 signaling by modulating the phosphorylation state of Rb could indirectly affect DNMT1 levels or activity. However, the inability of short or long term TGFβ1 treatment to induce MGMT or reduce MGMT methylation in the TGFβ1/−/− lines suggests that MGMT regulation by TGFβ1 is indirect and that hypermethylation, once achieved, is stable. Both the human and mouse MGMT promoters contain regions of high GC content and multiple SP1 sites (30, 36). A recent model for the evolution of hypermethylation in promoters suggests that SP1 sites protect GC-rich regions from spreading of methylation (43). It is intriguing that Smad3 and Sma4, intracellular mediators of TGFβ1 signaling, can activate transcription through interaction with SP1 proteins and SP1 DNA binding sites (44, 45). It is tempting to speculate that alterations in Smad levels or activity due to inhibition of TGFβ1 signaling could influence occupancy of SP1 sites and effect increased accessibility to de novo methylation.

In conclusion we have demonstrated distinct patterns of CpG methylation at the MGMT promoter in immortal keratinocyte lines, which differ by ability to produce autocrine TGFβ1. Hypermethylation of the MGMT promoter specifically in the TGFβ1/−/− keratinocytes occurs during immortalization and subsequent passages, suggesting that these cells, because of the absence of TGFβ1 signaling, are more susceptible to hypermethylation. Analysis of CpG islands of a number of genes will be required to determine if this is specific for the MGMT locus or represents a general methylator phenotype. However, our results also show that the MGMT promoter also becomes methylated during tumor development in the mouse multitissue skin carcinogenesis model (2), suggesting that this in vitro observation is relevant to neoplastic transformation of mouse keratinocytes in vivo. It will be important to determine if aberrant MGMT methylation contributes to either spontaneous or alkylating-induced mutation and transformation in the TGFβ1/−/− cells. Given that loss of expression of TGFβ1 is associated with malignant progression in the mouse skin tumor model (17) and that defects in TGFβ signaling occurs frequently in both human tumors and tumor cell lines (10, 11, 32), it is tempting to speculate that inactivation of the TGFβ pathway may play a causal role in the widespread hypermethylation of genes that occurs during cancer development and progression.

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Increased Sensitivity of Transforming Growth Factor (TGF) β1 Null Cells to Alkylating Agents Reveals a Novel Link between TGF β Signaling and Oβ6-Methylguanine Methyltransferase Promoter Hypermethylation

Hisaharu Yamada, Kinnimulki Vijayachandra, Carrie Penner and Adam Glick

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