Abstract
Basic fibroblast growth factor (bFGF) is a multifunctional protein and one of the most important growth factors in cutaneous melanoma development and progression. We hypothesized that high bFGF expression might be responsible for chemoresistance in advanced melanoma. M14 human melanoma cells expressing low levels of bFGF were successfully transfected with vectors encoding either the 18 kDa or all isoforms of bFGF. M14 cells and bFGF-overexpressing clones had a similar growth rate in vitro. Overexpression of 18 kDa or all isoforms of bFGF resulted in, respectively, 2.9- and 6.9-fold resistance against temozolomide. O6-Aldkyguanine-DNA-alkyltransferase (AGT) protein levels were highly elevated. Specific inhibition of AGT with O6-benzylguanine completely reversed the resistance in the 18 kDa clone, and partially in the clone overexpressing all isoforms. A methylation-specific PCR showed that at least in the 18 kDa overexpressing clone, increased AGT expression resulting in temozolomide resistance. Overexpression of all isoforms of bFGF, but not the 18 kDa isoform alone, resulted in 2.9-fold resistance against cisplatin, which could not be reversed by O6-benzylguanine. The expression levels of the mismatch repair proteins MSH2, MSH6, and MLH1 were not decreased, which likely excludes a defective mismatch repair system as a cause for cisplatin resistance. There were no changes in sensitivity to docetaxel and doxorubicin. In conclusion, bFGF overexpression can result in resistance against temozolomide mediated by demethylation of the O6-methylguanine-DNA-methyltransferase promoter. [Mol Cancer Ther 2007;6(10):2807–15]

Introduction
The treatment of melanoma, once it has metastasized, has a poor outcome. Few chemotherapeutic agents have shown activity in patients with melanoma metastases. The most well-known drug for the treatment of melanoma, the methylating agent dacarbazine, has a response rate of ~20%, but does not improve survival. A derivative of dacarbazine, temozolomide, has similar activity to dacarbazine, as shown in a phase 3 clinical trial in patients with melanoma (1). In contrast to dacarbazine, temozolomide is able to cross the blood–brain barrier and, as a result, has activity in melanoma brain metastases (2, 3). Temozolomide can be given orally and it spontaneously hydrolyzes to its active metabolite 5-(3,3-methyltriazen-1-yl)imidazole-4-carboxamide under alkaline conditions in blood and tissues (4).

The cytotoxicity of temozolomide is ultimately mediated by the O6 position of guanine to form O6-MeG DNA adducts. During DNA synthesis, the modified guanine preferentially pairs with thymine, thereby activating the mismatch repair (MMR) system. Several rounds of futile mismatch repair then lead to the induction of apoptosis in a highly efficient way (5). Temozolomide adducts are almost exclusively repaired by the enzyme O6-alkylguanine-DNA-alkyltransferase (AGT) encoded by the gene O6-methylguanine-DNA-methyltransferase (MGMT). AGT is unique among DNA repair proteins in that it functions alone, without the need for additional proteins or repair pathways. A strong correlation between AGT activity and resistance against methylating agents, such as temozolomide, has been shown in melanoma cells in vitro (6, 7). The MGMT gene is not commonly mutated or deleted. Instead, loss of MGMT transcription is mainly the result of hypermethylation of Cpg islands in the promoter resulting in decreased mRNA and protein expression and loss of enzymatic activity (8, 9).

In melanoma, basic fibroblast growth factor (bFGF) is the best characterized autocrine growth factor (10). In contrast to melanocytes, almost all melanomas produce bFGF protein. bFGF can act as a growth stimulator in every
sequential step of melanoma progression. The important role of bFGF as an autocrine growth factor has clearly been established in different studies: (a) neutralizing antibodies against bFGF could inhibit the growth of bFGF-producing melanoma cells (10), (b) melanoma cell growth was decreased by antisense oligodeoxynucleotides targeted to bFGF (11) or to FGF receptor-1 (12), (c) growth of melano-

noma xenografts could be completely arrested when tumors were injected with antisense bFGF or FGF receptor-1 cDNAs (13).

There is increasing evidence that bFGF can modulate sensivity to various anticancer agents, as observed in a number of immortalized cell lines in vitro as well as in vivo. 3T3 fibroblasts transfected with bFGF cDNA showed resistance against N-(phosphonacetyl)-L-aspartate, etoposide, 5-fluorouracil, as well as cisplatin (14–16). Miyake et al. (17) have described in a human bladder cancer cell line in vitro as well as in vivo, that expression of bFGF was associated with resistance against cisplatin.

Because bFGF is an important growth factor for melano-

noma, we hypothesized that chemosensitivity may be related to the amount of bFGF protein expressed. Translation of the bFGF gene could result in the formation of different isoform proteins (18). The 18 kDa protein is mainly localized in the cytoplasm, but can also be secreted in biological fluids and in plasma. It is bound to heparan sulfate proteoglycans on the cell membrane. Due to the expression of a nuclear localization sequence, the high-
molecular weight isoforms are localized in the nucleus. We designed a study to determine whether overexpression of the 18 kDa or all isoform proteins of bFGF in M14 melano-

noma cells would result in changes in chemosensitivity. Indeed, we observed that overexpressed bFGF induced resistance against the DNA-damaging agents temozolo-
mide and cisplatin, but not against doxorubicin and
docetaxel. We found that, at least in the 18 kDa–transfected clones, temozolomide resistance was due to increased AGT expression associated with demethylation of the MGMT promoter.

Materials and Methods

Drugs and Reagents

Temozolomide (Schering-Plough Research Institute) was dissolved in DMSO (Riedel-de Haën) to a final concentration of 100 mmol/L. Doxorubicin (Pharmachemie) was dissolved in water at a concentration of 3.45 mmol/L. A 1 mg/mL solution of cisplatin was purchased from Bristol-Myers Squibb. Docetaxel (Sanofi-Aventis) was dissolved in DMSO to a final concentration of 10.5 mmol/L. O6-Benzyglyguanine (O6-BG; Sigma-Aldrich Chemie) was dissolved in DMSO to 20.7 mmol/L. 5-Azacytidine (5-AzaC; Sigma-Aldrich) was diluted in PBS to a stock concentration of 1 mmol/L. Drugs were further diluted in tissue culture medium when investigated for their anti-

proliferative effects in vitro. The final concentration of DMSO did not exceed 1% (v/v), which was nontoxic to the cells.

Cell Culture

The human melanoma cell line M14 was a kind gift from Drs. J.R. Westphal and W. Leenders, University Medical Centre St. Radboud, Nijmegen, the Netherlands, and has been established from melanoma metastasis (19). The BRO cell line has been derived from a highly malignant and aggressive primary melanoma (20). Melanoma cell lines were cultured in DMEM (Bio-Whittaker) supplemented with 10% heat-inactivated FCS (Invitrogen), 50 units/mL of penicillin (ICN Biochemicals) and 50 μg/mL of strepto-
mycin (ICN Biochemicals). bFGF-overexpressing 1F6 clones were cultured in the presence of 500 μg/mL of genetin (Invitrogen) to maintain the selection of bFGF overexpression. Cells were grown at 37°C in humidified air containing 5% CO2.

Constructs and Stable Transfection

The cDNA of the 18 kDa and that of all isoform proteins of human bFGF were a kind gift from Dr. R.Z. Flikkiewicz, Pritzm Pharmaceuticals, Inc., San Diego, CA (18). The vectors CMV-bFGF-ALL, encoding for all isoforms, and pCMV-bFGF 18 kDa, encoding for the 18 kDa isoform, were obtained by cloning, respectively, the entire 1108 bp of cDNA or the 18 kDa cDNA into the EcoRI site of pcDNA3 (Invitrogen) under the control of the CMV immediate-early promoter. All vectors contained a genetin resistance gene to allow for the selection of positive clones. M14 cells were stably transfected by use of the calcium phosphate precipitation method as has been described (21). For the control pcDNA3 “empty” vector transfection, polyclonal clones were expanded. bFGF-overexpressing monoclonal clones M14-18 kDa 20.3 and M14-ALL 23.1 were selected for further experiments.

Isolation of RNA and Quantitative RT-PCR

Total RNA from cell lines was isolated using Trizol reagent (Invitrogen) according to the manufacturer’s pro-
tocol. Thirty micrograms of RNA was reverse-transcribed with 50 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen) in the presence of 1.2 μL of 0.5 μg/mL random primers.

Quantitative LightCycler PCR was used to confirm the overexpression of bFGF in transfected cells. bFGF mRNA was analyzed with the LightCycler-Faststart DNA Master Hybridization Probes kSYBR Green 1 kit (Roche Diagnosti-
s). The human bFGF (sense, 5′-TGTGCTAACCCT-

ACCTGGC-3′; antisense, 5′-ATAGGTTTC CCAGGTCC-3′), and, as an internal control, the human β2-microglobulin (sense, 5′-GATGAGTATGCCTGCCTG-3′; antisense, 5′-CAATCCAAATGCAGGCATCT-3′) genes were amplified according to the manufacturer’s protocol. In short, the reaction mix contained 3 mmol/L of MgCl2 (bFGF) or 4 mmol/L of MgCl2 (β2-microglobulin), 0.5 μmol/L of sense and antisense primer, 1× FastStart DNA Master SYBR Green 1 mix (containing LightCycler FastStart Enzyme, FastStart Taq DNA polymerase, SYBR green dye, deoxynucleotide triphosphates, and reaction buffer), and 2 μL of cDNA. PCR conditions were as follows: 95°C for 10 min followed by 40 cycles at 95°C for 10 s, 60°C for 10 s, and 72°C for 18 s. cDNA was replaced by PCR-grade
water as a negative control. The relative expression levels of different samples were calculated from bFGF crossing points normalized to β2-microglobulin. Relative mRNA expression was calculated by: 

\[ \frac{E^{\Delta C_{p} \text{ target gene}}}{E^{\Delta C_{p} \text{ reference gene}}} \]

in which \( E \) = efficiency and \( \Delta C_{p} = \) crossing point when compared with M14 or M14-pcDNA3 cells. For each experiment, melting curve analysis was done. Primer efficiencies were determined using pooled cDNAs.

**Western Blot**

Melanoma cell lines and bFGF-overexpressing clones were lysed in ice-cold FOS-RIPA lysis buffer (10 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% SDS, 0.1% NP40, and 0.1% sodium deoxycholate) supplemented with 0.5 mmol/L of trypsin inhibitor (Sigma-Aldrich), 0.5 μg/mL of leupeptin (Sigma-Aldrich), and 1 mmol/L of phenylmethylsulfonyl fluoride (Merck) for 10 min on ice. Lysates were centrifuged for 15 min at 13,000 rpm at 4°C. Protein concentrations of cell lysates were measured according to Bradford (22).

Proteins were denatured by addition of sample buffer containing β-mercaptoethanol and incubation at 95°C for 5 min. Proteins were subjected to SDS-PAGE on 12% polyacrylamide gels and electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore). Membranes were blocked for 1 h in TBST [10 mmol/L Tris (pH 8), 150 mmol/L NaCl, and 0.025% Tween 20] / 5% milk and incubated overnight with 0.2 μg/mL of rabbit polyclonal human bFGF-directed antiserum (Santa Cruz Biotechnology), 1:1,000 mouse monoclonal anti-MGMT antibody (MT 23.2; Abcam), 1 μg/mL of mouse monoclonal anti-MLH1 (clone 14; Zymed Laboratories), 1 μg/mL of mouse monoclonal anti-MSH2 (clone GB12; Oncogene Research Products), 1:500 mouse monoclonal anti-human β-actin (Sigma-Aldrich) in TBST / 5% milk. After washing with TBST, the membranes were incubated for 1 h at room temperature with horseradish peroxidase–conjugated mouse anti-rabbit IgG (1:2,000 dilution; Cell Signalling Technology) or rabbit anti-mouse IgG (1:1,500; DAKO, Heverlee, the Netherlands) in TBST / 5% milk. Membranes were washed and proteins were visualized by electrochemiluminescence.

**In vitro Chemosensitivity**

The antiproliferative effects of drugs were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay (Sigma-Aldrich) as described previously (23). Briefly, 3,000 cells per well in 200 μL of medium were plated in 96-well plates and grown for 24 h at 37°C. Cells were exposed for 1 h to serial dilutions of drugs in a total volume of 200 μL in four replicate wells. Thereafter, drugs were washed away and cells were incubated for an additional 96 h. The medium was removed and 50 μL of 3-(4,5-dimethylthiazol-2-yl)-2,6-dimethylmorpholin-2,5-diphenyl-tetrazolium bromide in PBS (0.4 μg/mL) was added. Plates were incubated for 2 h and formazan crystals formed were dissolved in 200 μL of DMSO. The absorbance was measured at 540 nm using a Labsystems Multiscan Bichromatic plate reader (Labsystems). Results were expressed as the IC\(_{50}\), which is the concentration of drug inducing 50% inhibition of cell growth as compared with control cell growth. The ratio between the IC\(_{50}\) value of transfected cells divided by the IC\(_{50}\) of M14 parent cells was designated as the resistance factor (RF).

In experiments designed to test the effect of O\(^6\)-BG on sensitivity, cells were pretreated for 2 h with 5 μmol/L of O\(^6\)-BG or complete medium. Then, anticancer drugs were added for an exposure time of 1 h. Thereafter, drugs and O\(^6\)-BG were washed away and cells were incubated in 200 μL of complete medium alone or with O\(^6\)-BG for an additional 96 h. Control cells were either untreated, or treated with O\(^6\)-BG alone to confirm the absence of toxicity.

**Transient Transfection and Dual Luciferase Activity Assays**

The MGMT promoter-firefly luciferase reporter construct p-954/+24ML was originally described by Dr. K. Bhakat, University of Texas Medical Branch, Galveston, TX (24, 25). The expression vectors pRenilla-thymidylate kinase (TK) and pGL2 were purchased from Promega. Exponentially growing M14 cells and bFGF-overexpressing M14 clones (2 × 10\(^5\)) were plated in six-well plates. After 24 h, cells were transiently transfected with 0.5 μg of each reporter plasmid using Fugene 6 transfection reagent (Roche Diagnostics) for 48 h following the manufacturer’s protocol. To correct for transfection efficiency, 50 ng of pRenilla-TK was cotransfected. The activities of firefly and Renilla luciferase were determined with the dual luciferase reporter assay (Promega) according to the manufacturer’s protocol. In short, cells were lysed by scraping in the presence of 250 μL of 1× passive lysis buffer. Twenty microliters of cell lysate was transferred into the luminometer tube containing 100 mL of LAR II (Promega), and firefly luciferase activity was measured. Then, Renilla luciferase activity was measured after adding 100 mL of Stop & Glo Reagent (Promega). Relative MGMT promoter activity in M14 and bFGF-transfected clones was calculated by the formula:

\[
\frac{(\text{MGMT firefly luciferase value})/(\text{pRenilla-TK luciferase value})}{(\text{pGL2 basic firefly luciferase value})/(\text{pRenilla-TK luciferase value})}
\]

**5-AzaC Treatment**

M14 cells were continuously exposed to 1 μmol/L of 5-AzaC over a period of 21 days. Every 2 days, cells were subcultured and fresh medium containing 5-AzaC was added. Cell lysates were prepared on days 0, 2, 4, 7, 9, 11, 14, 16, 18, and 21 after the start of drug exposure. AGT protein expression was determined by Western blot. The effect of 5-AzaC treatment on the sensitivity of M14 cells to temozolomide was determined in a 3-(4,5-dimethylthiazol-2-yl)-2,6-(dimethyl-morpholin)-2,5-diphenyltetrazolium bromide assay as described above.

**Methylation-Specific PCR**

MGMT promoter methylation was studied by a methylation-specific PCR. This method discriminates between
methylated and nonmethylated alleles of a given gene (26). In short, DNA was isolated using the Qiagen Spin Miniprep Kit (Qiagen) according to the manufacturer’s protocol. After the sodium bisulfite modification, the DNA was bisulfite treated with the EZ DNA methylation kit (Zymo Research) following the manufacturer’s protocol. Two micrograms of DNA was subjected to Miniprep Kit (Qiagen) according to the manufacturer’s protocol. Two micrograms of DNA was subjected to bisulfite treatment with the EZ DNA methylation kit (Zymo Research) following the manufacturer’s protocol. After the sodium bisulfite modification, the DNA was amplified in a total volume of 50 μL in the presence of specific primers for methylated (sense, TTTCGACGTTCG-TAGTTTTTCGC; antisense, GCACTCTTCCGAAAAC-GAAACG) and unmethylated (sense, TTTGTGTTTTGA-TGTGTGTGTTTAGTTTTGT; antisense, AACTCCACACT-CTTCCAAAAACAAAAA) MGMT promoter (8). The amplification conditions were as follows: the reaction was initiated by 7 min at 95°C, followed by 35 cycles at 95°C for 45 s, 58°C for 30 s, 72°C for 30 s, and ending by 7 min at 95°C. PCR products were separated on 2% agarose gels and visualized under UV illumination.

**Statistical analysis**

Statistical analysis of possible differences between mean values was done with a two-tailed Student’s t test. \( P < 0.05 \) was considered significant.

**Results**

**Chemosensitivity of bFGF-Overexpressing M14 Cells**

Recently, we described the characteristics of M14 melanoma cells stably transfected with constructs encoding for either the 18 kDa isoform alone, or all (ALL) isoforms of bFGF (21). bFGF mRNA and protein overexpression in M14 cells and clones is shown in Fig. 1. The BRO melanoma cell line is included because this is a cell line with high endogenous bFGF expression. Although 18 kDa and ALL isoforms of bFGF were highly overexpressed and confirmed to be biologically active in a human umbilical vein endothelial cell proliferation assay, no significant changes in *in vitro* as well as *in vivo* growth were observed. Mean *in vitro* cell doubling times (±SD) for M14, M14-pcDNA3, M14-18 kDa, and M14-ALL cells were 19.4 ± 1.4, 22.6 ± 1.3, 24.3 ± 4.1, and 23.7 ± 2.6 h, respectively, in medium containing 10% serum (21).

We determined the antiproliferative effects of temozolomide, cisplatin, doxorubicin, and docetaxel in M14 cells and bFGF-overexpressing M14 clones using a 1-h drug exposure time. A summary of the IC50 values obtained with the different drugs is given in Table 1. Although overexpression of either the 18 kDa or ALL isoforms of bFGF in M14 clones did not alter the sensitivity to doxorubicin and docetaxel, the sensitivity to temozolomide and cisplatin was significantly decreased. In M14-ALL cells, a RF of 2.7 was calculated for cisplatin (\( P < 0.05 \)), but no resistance against cisplatin was present in M14 cells overexpressing only the 18 kDa isoform of bFGF. The induction of resistance against temozolomide was evident in both M14-18 kDa (RF, 2.9; \( P < 0.05 \)) and M14-ALL cells (RF, 6.9; \( P < 0.05 \)).

**Table 1.** IC50 values for temozolomide, cisplatin, doxorubicin, and docetaxel in M14 cells and clones overexpressing the 18 kDa isoform of bFGF, all isoforms of bFGF, or transfected with a control pcDNA3 vector

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Temozolomide, IC50 μmol/L (±SD)</th>
<th>RF*</th>
<th>Cisplatin, IC50 μmol/L (±SD)</th>
<th>RF</th>
<th>Doxorubicin, IC50 μmol/L (±SD)</th>
<th>RF</th>
<th>Docetaxel, IC50 nmol/L (±SD)</th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>M14</td>
<td>58.3 (±12.6)</td>
<td>1</td>
<td>10.8 (±4.5)</td>
<td>1</td>
<td>0.52 (±0.27)</td>
<td>1</td>
<td>17.8 (±7.0)</td>
<td>1</td>
</tr>
<tr>
<td>M14-pcDNA3</td>
<td>81.3 (±19.3)</td>
<td>1.4</td>
<td>11.7 (±5.1)</td>
<td>1.1</td>
<td>0.29 (±0.04)</td>
<td>0.6</td>
<td>22.0 (±8.5)</td>
<td>1.2</td>
</tr>
<tr>
<td>M14-18 kD</td>
<td>370 (±10.0)</td>
<td>2.9</td>
<td>8.7 (±4.1)</td>
<td>0.8</td>
<td>0.49 (±0.02)</td>
<td>0.9</td>
<td>13.0 (±2.8)</td>
<td>0.7</td>
</tr>
<tr>
<td>M14-ALL</td>
<td>400 (±81.6)</td>
<td>6.9</td>
<td>29.0 (±5.7)</td>
<td>2.7</td>
<td>0.47 (±0.2)</td>
<td>0.9</td>
<td>21.3 (±5.9)</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*NOTE: IC50 values represent the drug concentration resulting in 50% growth inhibition as compared with control cells; mean values ± SD of at least three experiments are shown.

*RF is the ratio of IC50 clones/IC50 M14 parent cells.

*Significantly different (\( P < 0.05 \)) with reference to M14 and M14-pcDNA3.*
AGT protein nor temozolomide resistance (data not shown). AGT overexpression in M14-18 kDa cells was highly increased AGT protein levels were observed compared with that in M14 parent and M14-pcDNA3 cells. This suggests that, in these cells, monoallelic hypermethylation of the MGMT promoter was responsible for the lack of AGT protein expression.

To determine whether hypermethylation of the MGMT promoter in bFGF-overexpressing M14 clones would be responsible for the lack of AGT protein expression in M14 cells, we continuously treated M14 cells with the demethylating agent 5-AzaC for 22 days. Figure 3A shows that AGT expression gradually increased beyond 12 days of 5-AzaC treatment, indicating that hypermethylation of the MGMT promoter is a likely cause of the absence of AGT expression. Increased AGT expression in 5-AzaC-treated M14 cells resulted in a 3-fold resistance against temozolomide as compared with control M14 cells (P < 0.05; Fig. 3B). Addition of the AGT inhibitor O6-BG to 5-AzaC-treated M14 cells completely abrogated resistance, whereas the sensitivity of parent M14 cells was not affected. Thus, the observed resistance in M14 cells treated with 5-AzaC could be explained by increased AGT expression.

In order to determine, on the molecular level, whether demethylation of the MGMT promoter in bFGF-overexpressing M14 clones would be responsible for the increased AGT expression, we did a methylation-specific PCR. The human colon cancer cell lines Colo320 and HT-29 were included as positive controls for expressing only methylated or unmethylated MGMT promoter, respectively. In Fig. 4, it is illustrated that specific PCR products for both methylated and unmethylated CpG sites in the MGMT promoter were found in M14 and M14-pcDNA3 cells. This suggests that, in these cells, monoallelic hypermethylation is dominant. In M14-18 kDa cells, however, we only detected a specific product for the unmethylated allele encoding for enhanced AGT protein expression. Despite the increased AGT expression, we repeatedly did not find differences in the methylation pattern of M14-ALL cells as compared with that in M14 parent and M14-pcDNA3 cells.

Expression of AGT and Treatment with O6-BG
AGT is almost exclusively involved in resistance against temozolomide and its protein expression has been described to be closely correlated with its activity (27). Therefore, AGT protein expression levels were determined by Western blot in M14 parent and M14-pcDNA3 cells, hardly any AGT protein could be detected. In M14 clones overexpressing either 18 kDa or ALL isoforms of bFGF, highly increased AGT protein levels were observed (Fig. 2A). AGT overexpression in M14-18 kDa cells was more pronounced than that in M14-ALL cells. Continuous exposure of M14 cells to exogenous recombinant human bFGF at 20 ng/mL for 7 weeks, however, induced neither AGT protein nor temozolomide resistance (data not shown).

The AGT inhibitor O6-BG can restore sensitivity to temozolomide in a number of cell types with high expression of AGT. Recently, O6-BG treatment was also found to enhance cisplatin-induced toxicity in head and neck cancer cell lines independent of AGT activity (27). Therefore, we tested whether the resistance of bFGF-overexpressing M14 clones against temozolomide and cisplatin could be reversed by O6-BG (Fig. 2B). Treatment of M14-18 kDa cells with a nontoxic concentration of O6-BG (5 μmol/L) resulted in the complete restoration of sensitivity to temozolomide (P < 0.05). For M14-ALL cells, we observed a partial recovery of sensitivity (P < 0.05), suggesting that additional mechanisms of resistance against temozolomide could play a role. Treatment of control cells did not influence the sensitivity to temozolomide. We did not observe any reversal of resistance against cisplatin in M14-ALL cells, indicating that increased AGT expression could not explain cisplatin resistance.

Demethylation by 5-AzaC and Methylation-Specific PCR
To determine whether hypermethylation of the MGMT promoter was responsible for the lack of AGT protein expression in M14 cells, we continuously treated M14 cells with the demethylating agent 5-AzaC for 22 days. Figure 3A shows that AGT expression gradually increased beyond 12 days of 5-AzaC treatment, indicating that hypermethylation of the MGMT promoter is a likely cause of the absence of AGT expression. Increased AGT expression in 5-AzaC-treated M14 cells resulted in a 3-fold resistance against temozolomide as compared with control M14 cells (P < 0.05; Fig. 3B). Addition of the AGT inhibitor O6-BG to 5-AzaC-treated M14 cells completely abrogated resistance, whereas the sensitivity of parent M14 cells was not affected. Thus, the observed resistance in M14 cells treated with 5-AzaC could be explained by increased AGT expression.

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MGMT Promoter Activity in bFGF-Overexpressing M14 Clones
Another possibility for the increased AGT expression in bFGF-overexpressing M14 clones would be the activation of the MGMT promoter, resulting in increased transcription. To explore this phenomenon, we did transient transfection experiments with the full-length MGMT promoter (p-954/+24ML) coupled to luciferase. As can be...
seen in Fig. 5, no induction of MGMT-954/+24ML luciferase activity was observed in M14-18 kDa or M14-ALL clones. Therefore, we could rule out that the increased AGT expression in bFGF-overexpressing M14 clones was regulated through the induction of the MGMT promoter activity.

**MMR Protein Expression**

The MMR pathway is critically involved in the induction of DNA damage by temozolomide, but also by cisplatin. A reduction in protein expression of one or more of the proteins constituting this pathway has been shown to result in increased resistance against both drugs. Therefore, we compared the protein expression levels of three major MMR proteins, MLH1, MSH2, and MSH6, between M14 parent cells and bFGF-overexpressing clones. We did not find altered expression levels of these proteins (Fig. 6), suggesting that major changes were not involved in the resistance against temozolomide and cisplatin.

**Discussion**

We are the first to show that melanoma cells stably transfected with bFGF could develop resistance against cisplatin and temozolomide, agents known to directly damage DNA. Sensitivity to docetaxel and doxorubicin was not affected. Previous studies in NIH-3T3 cells and tumor cells have indicated that bFGF can be involved in the resistance against a variety of unrelated anticancer agents (14–17). bFGF-induced resistance against cisplatin has been described in bFGF-transfected NIH-3T3 cells which coincided with elevated Mdm2 and reduced activation of p53 (15). Overexpression of bFGF in a human bladder cancer cell line resulted in a 3- to 4-fold increased resistance against cisplatin not only in vitro, but also in vivo (17). In melanoma cells, however, the role of bFGF in chemoresistance has not been previously evaluated. In addition, bFGF-induced resistance against temozolomide has not yet been described.

Dini et al. (16) have determined the role of the different bFGF isoform proteins in N-(phosphonacetyl)-L-aspartate resistance in NIH-3T3 fibroblasts. Overexpression of the high–molecular weight bFGF as well as all isoform proteins resulted in higher resistance against N-(phosphonomethyl)glycine as compared with the overexpression of 18 kDa alone. These results are in accordance with our study, in which we show that M14-ALL cells were more resistant against temozolomide than M14-18 kDa cells. In addition, resistance against cisplatin was only observed in M14-ALL cells and not in M14-18 kDa cells. Exogenous recombinant human bFGF did not affect M14 sensitivity to temozolomide. Together, our data and those of the group of Dini et al. (16), show a critical role for the high–molecular weight bFGF isoform proteins in the induction of drug resistance.

In general, high expression of the DNA repair enzyme AGT correlates with resistance against temozolomide. High levels of AGT have been observed in a number of human tumors, including melanoma, colon cancer, pancreatic cancer, lung cancer, and gliomas (28–35). We found that although AGT protein was not expressed in M14 and M14-pcDNA3 cells, AGT levels were highly increased in temozolomide-resistant cell clones M14-18 kDa and M14-ALL. Because the AGT inhibitor O6-BG completely restored sensitivity in M14-18 kDa cells, enhanced AGT expression was responsible for temozolomide resistance. O6-BG only
partially sensitized M14-ALL cells, which indicates the presence of additional resistance mechanisms. As expected, treatment with Oβ-BG did not change the antiproliferative effects of cisplatin in both M14-18 kDa and M14-ALL cells. Although Fishel et al. (27) have shown that pretreatment of head and neck cancer cell lines with Oβ-BG enhanced the cytotoxicity of cisplatin and carboplatin, this observation was independent of AGT expression.

High AGT expression levels are clearly involved in temozolomide resistance, but some cell lines with low AGT expression are nevertheless resistant. A deficiency in the MMR system due to mutation or loss of one of the MMR proteins may also be implicated in resistance as described for temozolomide, cisplatin, doxorubicin, and etoposide (36). Lage et al. (37) have reported that the nuclear content of the MMR proteins MLH1, MSH2, and MSH6 was reduced by up to 80% in melanoma cell lines resistant against cisplatin, etoposide, and vindesine. Pepponi et al. (7) have shown that, in MMR-proficient melanoma cells, a clear correlation existed between AGT expression and temozolomide sensitivity, which underlines an intact MMR system to be essential for temozolomide to be active. MMR-deficient cells were resistant against temozolomide irrespective of their AGT expression. We did, however, not detect any changes in protein expression levels of the major MMR proteins MSH2, MLH1, and MLH6 in M14-ALL cells. In both M14 and M14-pcDNA3, we found specific bands for both methylated and unmethylated MGMT responsible for the induction of AGT expression. In a study by Lind et al. (40) the methylation profile of primary colorectal carcinomas and colon cancer cell lines was biallelic in most cases, meaning that either methylated or unmethylated bands were detected. In 10 out of 20 cell lines, however, the methylation pattern was monoallelic because both methylated and unmethylated fragments were present. It has been observed that hypermethylation can selectively silence individual alleles in malignant cells (41, 42). Because our M14 cells contain both methylated and unmethylated MGMT bands, allele-specific methylation may have caused silencing of the gene. Oβ-BG treatment only partially reversed AGT-mediated resistance against temozolomide in M14-ALL cells. The presence of both unmethylated and methylated MGMT bands in M14-ALL cells indicated that the MGMT

in endothelial cells, both ectopic and exogenous bFGF could regulate the expression of the nucleotide excision repair proteins GADD45 and HMG-1. In addition, the 24 kDa isoform of bFGF was found to increase the expression and activity of DNA-dependent protein kinase, which is involved in the detection and repair of DNA double-strand breaks induced by ionizing radiation. The possible role of these nucleotide excision repair proteins and others, such as ERCC1, in cisplatin resistance in our bFGF-overexpressing M14 clones deserves further investigation.

AGT expression is mainly regulated by the methylation of its gene MGMT. This methylation occurs on the cytosine of CpG islands in the promoter and is mediated by 5′-methyltransferase. A number of studies have indicated that methylation of the MGMT promoter is associated with the absence of AGT mRNA and protein expression and activity (8, 9, 39). Furthermore, treatment with demethylating agents, such as 5-AzaC, reactivates transcription and leads to the restoration of AGT activity exemplified in M14 cells. In both M14 and M14-pcDNA3, we found specific bands for both methylated and unmethylated MGMT responsible for the induction of AGT expression. In a study by Lind et al. (40) the methylation profile of primary colorectal carcinomas and colon cancer cell lines was biallelic in most cases, meaning that either methylated or unmethylated bands were detected. In 10 out of 20 cell lines, however, the methylation pattern was monoallelic because both methylated and unmethylated fragments were present. It has been observed that hypermethylation can selectively silence individual alleles in malignant cells (41, 42). Because our M14 cells contain both methylated and unmethylated MGMT bands, allele-specific methylation may have caused silencing of the gene. Oβ-BG treatment only partially reversed AGT-mediated resistance against temozolomide in M14-ALL cells. The presence of both unmethylated and methylated MGMT bands in M14-ALL cells indicated that the MGMT

Figure 5. MGMT promoter activity in M14, M14-pcDNA3, and bFGF-overexpressing clones. One microgram of MGMT-954/+24ML was cotransfected with 50 ng of pRenilla-TK. Values represent MGMT luciferase activity divided by pRenilla-TK activity of duplicate samples from two independent experiments (columns, means; bars, range).

Figure 6. Expression of MMR proteins MLH1, MSH2, and MSH6 in M14 parent, M14-pcDNA3, and bFGF-overexpressing clones as determined by Western blot. Fifty micrograms of total protein was loaded on SDS-PAGE gels and protein bands were detected by specific antisera. Western blot is representative for three independent experiments. β-actin was used as a loading control.
functional gene was not fully recovered. Increasing evidence suggests that complete repression of the MGMT gene requires the methylation of CpG sequences in both promoter and neighboring regions of the gene (43). Possibly, bFGF is also involved in the demethylation of other sites in the gene, thereby releasing the chromatin structure and enhancing the binding of transcription factors to the promoter. Incomplete demethylation might explain the fact that because AGT expression is up-regulated to a lesser extent in M14-ALL cells compared with M14-18 kDa cells, we did not find a change in promoter methylation. In addition to methylation, AGT expression can also be regulated by activation of its promoter. The MGMT promoter includes six Sp1 binding sites within the CpG island, two glucocorticoid-responsive elements and two activator protein-1 and activator protein-2 binding sites (39). MGMT mRNA expression in HeLa cells could be up-regulated by phorbol-12-myristate-13-acetate and diacylglycerol, both activators of protein kinase C, which may be mediated by the transcription factor activator protein (44). In addition, Bhakat and Mitra (24) have reported that MGMT promoter activation by trichostatin A was dependent on the presence of two activator protein binding sites, whereas ectopic expression of the transcriptional coactivators p300 and cyclic AMP response element-binding protein enhanced this effect. By transfecting M14 and bFGF-overexpressing clones with a luciferase-MGMT promoter construct, we did not find changes in promoter activity, thus excluding a role for bFGF-mediated MGMT promoter activation.

Because almost all progressive melanomas produce high amounts of bFGF, it can be speculated that inhibition of bFGF signaling would not only abrogate autocrine and paracrine bFGF-stimulated growth, but would also increase the sensitivity to temozolomide by down-regulating AGT. In a human melanoma xenograft model, it has already been shown that prolonged administration of the combination of O6-BG and temozolomide resulted in greater antitumor effects compared with temozolomide alone (45, 46). Presently, small molecules that inhibit receptor tyrosine kinases, among which are FGF receptors, are being explored for usefulness in cancer treatment. It should be investigated whether compounds interfering with bFGF signaling will sensitize melanomas to DNA-damaging agents, such as temozolomide and cisplatin.

References


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