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Role of O^6-Methylguanine-DNA Methyltransferase in the Resistance of Pancreatic Tumors to DNA Alkylating Agents

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ABSTRACT

Pancreatic adenocarcinomas rarely respond to radiation or chemotherapy, indicating that a large percentage of these tumors possess complex mechanisms of resistance. The failure of alkylating agents, such as carmustine [1,3-bis(2-chloroethyl)-1-nitrosourea; BCNU], lomustine [1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; CCNU], and streptozotocin, to yield consistent therapeutic results further suggests that one of these mechanisms may be the high expression of O^6-methylguanine-DNA methyltransferase (MGMT). All 12 human pancreatic ductal adenocarcinomas assayed for MGMT activity showed unusually high levels, implying that these malignancies are efficient in repairing genotoxic O^6-alkylguanine lesions induced by methylating (streptozotocin) and 2-chloroethylating (BCNU and CCNU) chemotherapeutic genotoxic agents. Immunohistochemical analysis of an additional 15 pancreatic tumors showed that high levels of MGMT protein reside in the nucleus and the cytoplasm of malignant cells. Both nuclear and cytoplasmic staining were absent in hyperplastic duct epithelium, but staining was invariably present in moderate to highly dysplastic foci and especially strong in invasive components of the tumor. With the exception of lymphocytes that were MGMT positive, acinar, ductal, and islet cells did not stain for MGMT in histologically normal pancreata. These data indicate that MGMT activity is up-regulated in dysplastic epithelium, and its expression increases during tumor progression, reaching the highest levels in the invasive components of the tumor. Resistance of pancreatic tumor cells to alkylating agents was verified with four pancreatic tumor cell lines. CAPAN-2, CFPAC-1, PANC-1, and MIAPaCa-2, having MGMT levels of 1800, 987, 700, and 880 fmol/mg protein, respectively, were resistant to BCNU, but their resistance declined sharply following pretreatment with the MGMT inhibitor O^6-benzylguanine (O^6-BG). On the other hand, PANC-1 and MIAPaCa-2 could not be eradicated with *N*-methylnitrosourea (MNU) at concentrations as high as 2 mM, even when pretreated with O^6-BG. These two lines were shown to be modified genetically in microsatellite sequences by MNU and are believed to have a defective mismatch repair system, which may explain their resistance to methylating agents. Failure of pancreatic tumors to respond to nitrosoureas is related to high levels of MGMT expression and in some cases to genomic instability. However, these tumors can be sensitized to chloroethylating drugs and eradicated following the elimination of MGMT activity by O^6-BG or homologous MGMT inhibitors.

INTRODUCTION

In spite of its low incidence of only 0.01% (26,300 new cases diagnosed in 1996), pancreatic cancer remains the fifth leading cause of death from cancer in North America (1, 2). A cure is only possible by surgical removal of the tumor, but in practice, even this is rarely achieved because most tumors are no longer resectable at the time of diagnosis (3, 4). Fifteen to 20% of patients with carcinoma of the head of the pancreas can be resected, with about 14–33% of them surviving more than 5 years (5). Theoretically, the rate of survival could be improved further by intensifying surveillance of high-risk groups. However, lack of understanding of the etiology of pancreatic cancer (5) obscures identification of such groups and further impedes the development of preventive measures. A rising incidence of pancreatic cancer, at a rate of 1% per year since 1937, combined with our failure to prevent, diagnose, or treat pancreatic cancer successfully, makes a powerful case for further understanding of the biology of this disease as related to the development of more potent drugs for its treatment (6–10).

Pancreatic cancer, seen at the time of diagnosis, is usually a disseminated disease (11–13), and therefore, surgery alone is unlikely to increase survival rates in the absence of standard and well-tested adjuvant therapies. Present and future strategies for treatment should include the addition of adjuvant modalities or the development of new agents with significantly greater antitumor activities. Thus far, the results obtained with cytotoxic chemotherapy have been disappointing. More than 40 Phase II studies of many new agents and combinations have been reported in the last 15 years (14, 15). Among the more vigorously tested drugs are streptozotocin in combination with mitomycin; cisplatin in combination with Ara-C; 5-fluorouracil in various combinations with CCNU[2]; and methyl-CCNU with streptozotocin, mitomycin C, doxorubicin, cisplatin, methotrexate, cyclophosphamide, and vincristine (16–22). Treatment with 5-fluorouracil, doxorubicin, and mitomycin has shown a response varying between 10 and 34%, with a median survival of 10 months. All other treatments elicit a response lower than 20%, thus failing the standard for further testing. A newer drug, gemcitabine (2′,2′-difluorodeoxycytidine), with advanced cytotoxic properties in the laboratory, has induced a partial response in 5 of 44 patients (11%), with a median survival of 5.6 months and with 32% of the patients alive at 1 year (7, 23). The failure of a large number of chemotherapeutic agents against pancreatic tumors is consistent with the presence of a multidrug-resistant phenotype (not necessarily conferred by the p21 glycoprotein) in a large fraction of these tumors, but also it may be due to the relatively poor systemic access of several drugs to this organ (24, 25).

Of particular interest is the resistance of pancreatic tumors to chloroethylating nitrosoureas and other genotoxic agents. This could be attributed to effective repair of critical cytotoxic DNA lesions induced by such agents. One of the most supported mechanisms of tumor resistance to DNA-damaging agents is the overexpression of the repair protein MGMT (26–35). This protein rapidly reverses formation of adducts at the O^6 position of guanine and averts the formation of lethal DNA cross-links induced by chloroethylating nitrosoureas (36–38). MGMT is the major mechanism of resistance of tumor cells to bifunctional nitrosoureas, and its depletion with suicide inhibitors, such as O^6-BG, has been shown repeatedly to sensitize

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[2] The abbreviations used are: CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (lomustine); IPMC, intraductal papillary mucinous carcinoma; MGMT, O^6-methylguanine-DNA methyltransferase; MeG, methylguanine; O^6-BG, O^6-benzylguanine; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea (carmustine); MNU, *N*-methylnitrosourea; MMR, mismatch repair; MIN, microsatellite instability; UTSWMC, University of Texas Southwestern Medical Center.

tumor cells to BCNU (39–42). In this communication, we examined the capacity of pancreatic tumors to express MGMT and the effect of such expression on the resistance of pancreatic tumors to DNA methylation and chloroethylation by MNU and BCNU, respectively.

MATERIALS AND METHODS

Procurement of Tissue. Twelve frozen samples of randomly selected pancreatic tumor tissue were obtained from the University of Kentucky Hospital. Eight additional samples were obtained fresh, following Whipple or total pancreatectomies performed at UTSWMC. Seemingly normal tissue resected from sites remote from the tumor was available for four of the University of Kentucky Hospital tumors. Normal pancreatic parenchyma was also collected from all tumors obtained at UTSWMC. In addition, seemingly normal ductal epithelium and hyperplastic epithelium were obtained from the main pancreatic duct of pancreata removed at the UTSWMC site. Part of each sample was shock frozen no later than 0.5 h after resection by immersing it in liquid nitrogen, where it was stored until used, whereas another part was fixed in formalin and processed for histological examination.

Preparation of Tissue and Cell Extracts. Frozen tissues were cut in thin sections with a razor and immediately immersed in 50 mM cold (0°C) Tris-HCl (pH 8.0) containing 1 mM EDTA, 1 mM β-mercaptoethanol and 0.2% soybean trypsin inhibitor (homogenizing buffer). Sections containing necrotic or hemorrhagic regions were cut off under a magnifying scope and discarded; the remainder was homogenized on ice using an Ultraturrax (Omni, Waterbury, CT) at 15,000 rpm and subsequently sonicated on ice using a Branson Sonicator (Branson, Danbury, CT). Homogenates were centrifuged at 10,000 rpm for 10 min at 4°C, and clear supernatants were frozen in liquid nitrogen until assayed. Specimens from the main duct were cleaned from the surrounding tissue, and the duct was cut in a section of approximately 1 cm and inverted using a stainless steel wire. The surface of the duct was scraped with a scalpel, and the material removed was suspended in homogenizing buffer. Cell extracts were prepared by lysing cells in homogenizing buffer, thawing to room temperature, refreezing in liquid nitrogen (three times), and subsequently sonicating at 70% maximum output for 5 s (three times). Cell lysates were centrifuged at 15,000 × *g*, and supernatants were kept frozen in liquid nitrogen until use.

Biochemical Determination of MGMT. MGMT activity was determined, as described previously (43), by high-performance liquid chromatography analysis of the [^{3}H-CH$_3$]O^6-MeG content in acid hydrolysates of [^{3}H-CH$_3$]MNU methylated calf thymus DNA following incubation of the DNA with tissue or cell homogenates. In this assay, the detection limit for MGMT is dependent on the specific activity of the O^6-MeG in the DNA substrate. The substrate used had a specific activity of 16 Ci/mmol, and the detection limit for MGMT was approximately 5 fmol/mg protein. Protein determination was made using the Bio-Rad assay and corrected for the presence of trypsin inhibitor in the samples.

Statistical Analysis. Comparisons among groups of tumors in terms of MGMT activity were done by one-way ANOVA using Instat (Graph Pad, San Diego, CA).

***In Situ* Immunohistostaining for MGMT.** Fifteen tumors were examined immunohistologically using the anti-MGMT monoclonal antibody MT23.2, which was developed originally by Dr. Thomas Brent (St. Jude Children's Research Hospital) and Darell Bigner (Duke University) and donated generously by Dr. Brent. All immunostaining was performed at room temperature on a BioTek Solutions TechMate 1000 automated immunostainer (Ventana BioTek Systems, Tucson, AZ). Buffers, blocking solutions, secondary antibodies, avidin-biotin complex reagents, chromogen, and hematoxylin counterstain were used as supplied in the ChemMate secondary detection kit (Ventana BioTek Systems). Paraffin sections were cut at 3-μm intervals on a rotary microtome, mounted on positively charged glass slides (POP100 capillary gap slides, Ventana BioTek Systems), and air dried overnight. Sections were then deparaffinized in xylene and ethanol and placed in 200 ml of heat-induced epitope retrieval buffer (pH 6.8; Ventana BioTek Systems). The buffer was heated to the boiling point, after which 50 ml of deionized water were added. The buffer was again heated to the boiling point for 5 min, and then the slides were cooled in buffer for 20 min, after which they were rinsed thoroughly in deionized water followed by buffer. Sections were incubated in unlabeled blocking antibody solution for 5–10 min either with primary antibody at various dilutions ranging from 1:200 to 1:800 in buffer or with buffer alone, as a negative reagent control. After being washed in buffer, sections were incubated for 25 min with biotinylated polyvalent secondary antibody solution (containing goat antibodies to rabbit, mouse, and rat immunoglobulin). After another buffer wash, sections were incubated with three changes, 2.5 min each, of 3% hydrogen peroxide to inhibit endogenous tissue peroxidase activity and washed again in buffer. Sections were incubated for 25 min with freshly prepared horseradish peroxidase-conjugated avidin-biotin complex and then washed in buffer. The sections were incubated with three changes, 5 min each, of a freshly prepared mixture of diaminobenzidine and H_2O_2 in buffer, followed by washing in buffer and then water. Sections were counterstained with hematoxylin, dehydrated in a graded series of ethanol and xylene, and coverslipped. Slides were viewed by light microscopy. Positive reactions to diaminobenzidine were identified as dark brown reaction product. Sections were photographed on a Nikon Optiphot microscope (Nikon Instruments, Melville, NY).

Cell Growth and Cytotoxicity Assays. Four cell lines, CAPAN-2, CFPAC-1, MIAPaCa-2, and PANC-1, isolated originally from pancreatic ductal adenocarcinomas, were used in this study. All cell lines were maintained in Eagle's MEM (Life Technologies, Inc.) supplemented with lysine, valine, and leucine (100 μM each) and with 10% dialyzed fetal bovine serum. The medium was also supplemented with nonessential amino acids (1:100 dilution of stock from Life Technologies, Inc.), 1 mM sodium pyruvate, sodium bicarbonate, 6 μM α-hydroxycobalamin, 100 μM folic acid, 0.2 mg/ml gentamicin, and 100 μM L-methionine. The cytotoxicity of BCNU on cultured cells was measured as follows: cells growing at an exponential rate (10–20% confluency) in 100-mm Petri dishes were incubated at 37°C, either in medium alone or in medium containing 20 μM O^6-BG for 2 h. O^6-BG was synthesized and donated to us by Dr. Robert Moschel (National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD). Subsequently, dishes were washed with PBS and incubated with freshly prepared BCNU in PBS (pH 7.4) at concentrations ranging between 0 and 500 μM for an additional 45 min. Afterward, the BCNU was washed off with PBS, and the cells were incubated with medium containing 5 μM O^6-BG for 4 days. The cells were then trypsinized and counted using a Coulter Counter (Coulter Electronics, Hialeah, FL). The experiment was repeated using MNU at concentrations ranging between 0 and 2000 μM. The concentration of BCNU or MNU that halved the growth rate of the tumor cells (IC_{50}) was determined from plots of drug concentration *versus* the percentage of change in cell numbers as compared with untreated, nonconfluent controls 4 days after treatment. Conditions were optimized for the cells used in this study by exposure to BCNU and MNU in PBS, because the presence of serum or even nutrients yielded nonconsistent data. Also, incubations with nitrosoureas were done on attached cells and were limited to only 45 min, because three of four of the cultured pancreatic cells began to detach 1 h after the medium was replaced with PBS. Cells were not exposed to O^6-BG during treatment with BCNU or MNU, because nitrosoureas alkylate the MGMT inhibitor at rates sufficient to cause a reduction of their effective concentration.[3]

Determination of Mitosis and Apoptosis. Trypsinized cells derived from a 24–48-h treatment with BCNU or MNU were washed with PBS three times and spread on slides with a cytospin centrifuge at 500 rpm for 1 min. The mitotic index (mitosis per 1000 cells) was determined from a set of slides stained with H&E. Apoptosis was determined *in situ* on another set of slides using terminal deoxynucleotidyl transferase-mediated nick end labeling (Oncor, Gaithersburg, MD). Apoptosis was distinguished from necrosis by morphological characteristics, *i.e.*, condensed nuclei and nuclear fragmentation.

MIN. Three pancreatic tumor cell lines, CFPAC-1, MIAPaCa-2, and PANC-1, were negative for MIN during the course of 1 year of culturing. To evaluate possible loss of fidelity in DNA replication following treatment with MNU, cell lines were exposed to MNU alone (500 μM to 2 mM) for 45 min or to the same concentrations of MNU after they were treated with 20 μM O^6-BG for 2 h. O^6-BG-treated cells were subsequently cultured in medium supplemented with 5 μM O^6-BG for 4 days and after that in regular medium. Controls (no O^6-BG or MNU) were also cultured in parallel. Analysis of MIN was

[3] D. M. Kokkinakis, unpublished observations.

performed on DNA extracted from cultures treated with MNU alone, MNU plus O^6-BG [following several (>10) cycles of division], and also from controls. The following six loci were screened for MIN: *D2S123, D2S136, D3S1067, D5107, D6S87,* and *D18S34*. Primers for the amplification of these loci were purchased from Research Genetics, Inc. (Huntsville, AL). PCR amplification was performed using ^{32}P-end-labeled sense primers. The PCR product was denatured and electrophoresed on urea polyacrylamide (8%) gels. After electrophoresis, the gels were dried and visualized by autoradiography. Instability was determined as an alteration in banding patterns in MNU or MNU plus BG-treated cells as compared to matching nontreated (control) cells. After treatment, cells were washed with PBS and cultured in medium alone (MNU-treated) or medium containing 5 μM O^6-BG (MNU/O^6-BG treated) until they were confluent.

RESULTS

Patient Characteristics. The median age at diagnosis of patients with ductal adenocarcinomas in the pancreas was 57 years (range, 24–68 years). In addition to ductal adenocarcinomas, one patient had a tumor in the ampulla, one had a mucinous cystic adenocarcinoma, and two had a borderline mucinous cystic tumor. In addition, four cases of intraductal papillary mucinous carcinoma were identified.

MGMT Activity. A total of 20 pancreatic neoplasms, 12 segments of seemingly normal pancreatic parenchyma, and 6 samples of the seemingly normal main pancreatic duct epithelium were assayed for MGMT activity (Table 1). MGMT activity in pancreatic neoplasms was highly variable. Higher activity was observed in adenocarcinomas (mean, 459 fmol/mg protein; range, 160–875 fmol/mg protein; $n = 12$), followed by other less common tumors such as mucinous cystic tumors and IPMCs. The difference in MGMT levels between IPMCs and mucinous cystic tumors (cases 14–20), on one hand, and grade 3 ductal adenocarcinomas (cases 1–10), on the other, was statistically significant ($P = 0.0006$). No correlation was found between the age or sex of the patient and MGMT levels in the tumor. Unlike MGMT activity in tumors, MGMT activity in both normal parenchyma (mean, 41 fmol/mg protein; range, 29–66 fmol/mg protein; $n = 12$) and the seemingly normal main duct (mean, 35 fmol/mg protein; range, 32–46 fmol/mg protein; $n = 6$) was low in those patients from whom histologically normal tissue was available. The results suggest a correlation between tumor grade and level of MGMT in both adenocarcinomas and IPMCs.

Immunohistochemistry. Fifteen ductal carcinomas examined immunohistochemically showed intense nuclear and cytoplasmic staining with the MT23.2 MGMT monoclonal antibody. Highly dysplastic ductal cells stained in all 12 tumors, as shown in Fig. 1*A* and *B*, whereas hyperplastic ductal epithelium showed no reactivity or stained weakly (Fig. 1, *C* and *D*). Invasive carcinomas usually showed more intense staining than did noninvasive components of the same tumor (Fig. 1*E*). Cells staining for MGMT were only focally positive in the more differentiated areas of intraductal papillary carcinomas (Fig. 1, *F* and *G*) and varied in MGMT content as it was assessed from the intensity of staining. A more uniform and intense staining was observed in less-differentiated ductal carcinomas (Fig. 1*H*). Histologically normal pancreata did not usually stain. An exception was found in 2 specimens (of 12), in which islets and acinar cells trapped in the tumor showed variable nuclear staining (data not shown). Likewise, normal lymphocytes present in the tumor stroma and in adjacent lymph nodes also showed positive reactivity (data not shown). No reactivity was observed when the antibody was omitted (Fig. 1*I*). It must be noted that all tissue sections were processed simultaneously and subjected to the same treatment conditions. Failure to stain does not preclude the presence of MGMT, which may be present at an undetectable level. This is supported further by the biochemical detection of MGMT in normal ductal epithelium. On the basis of strong positive staining of Daoy tumor xenografts (MGMT, 320 fmol/mg protein) but not of U-138 xenografts containing less than 160 fmol/mg protein, we conclude that the cutoff point for positive detection of MGMT by our method lies between these two values.

Cell Lines. Four pancreatic tumor cell lines tested were all positive for MGMT activity. Levels of MGMT measured from 790 to 1800 fmol/mg protein (Fig. 2), a range that was significantly higher than the mean found in pancreatic adenocarcinomas. In addition, Daoy, a human medulloblastoma line, and U-263, a human glioblastoma line, which are well characterized in terms of MGMT expression and resistance to alkylating agents in our laboratory, were used for comparative purposes. Rates of growth for these lines measured as doubling time in our medium were 22 h for Daoy; 28 h for CFPAC-1, PANC-1, and U-263; 36 h for CAPAN-2; and 48 h for MIAPaCa-2. The rates were not affected by the inclusion of 5 μM O^6-BG in the medium for 4 days.

MGMT Inactivation by O^6-BG. As shown in Fig. 2, CAPAN-2 expressed 1800 fmol/mg protein MGMT activity. CFPAC-1, PANC-1, and MIAPaCa-2, expressed approximately one-half to one-third of this activity. Daoy and U-263 expressed 380 and 160 fmol/mg

Table 1 *MGMT activity in pancreas tumors*

Patient	Age (yr)	Sex	Type of surgery	Survival (months)	Tumor				Molecular alterations[b]	MGMT (fmol/mg/protein)
					Location	Histological type[a]	Stage	Grade		
1	62	M	Whipple	6	Head	DAC	3	3	—	442
2	58	F	Whipple	8	Head	DAC	3	3	—	511
3	61	M	Whipple	6	Head	DAC	4	3	Ki-ras	527
4	65	F	Whipple	4	Head	DAC	4	3	Ki-ras	611
5	44	M	Whipple	10	Head	DAC	4	3	MIN$^+$	307
6	68	M	Whipple	14	Head	DAC	3	3	MIN$^+$	875
7	62	M	Whipple	12	Head	DAC	3	3	MIN$^+$	460
8	24	F	Whipple	>52	Tail	DAC	3	3	MIN$^-$	350
9	62	M	Whipple	14	Head	DAC	3	3	MIN$^+$	211
10	64	F	Whipple	>6	Head	DAC	3	2	–	770
11	66	F	Whipple	14	Body	DAC	2	2	—	445
12	62	M	Gastrojejunostomy	4	Head	DAC	4	1	–	160
13	49	F	Whipple	>39	Ampullary	Adenocarcinoma	4	2	–	359
14	63	F	Distal pancreatectomy	1	Tail	Mucinus cystic borderline	1	2	–	135
15	41	F	Distal pancreatectomy	>5	Tail	Mucinus cystic borderline	1	1	–	145
16	47	M	Pancreatectomy	>7	Head	DAC-Chronic pancreatitis	1	1	–	95
17	68	M	Whipple	>24	Head	IPMC	1	1	–	211
18	54	F	Whipple	>18	Head	IPMC	1	2	–	185
19	52	M	Whipple	>84	Head	IPMC	1	1	—	130
20	63	F	Whipple	>12	Head	IPMC	1	2	—	240

[a] DAC, ductal adenocarcinoma; IPMC, intraductal papillary mucinous carcinoma.
[b] —, not done.

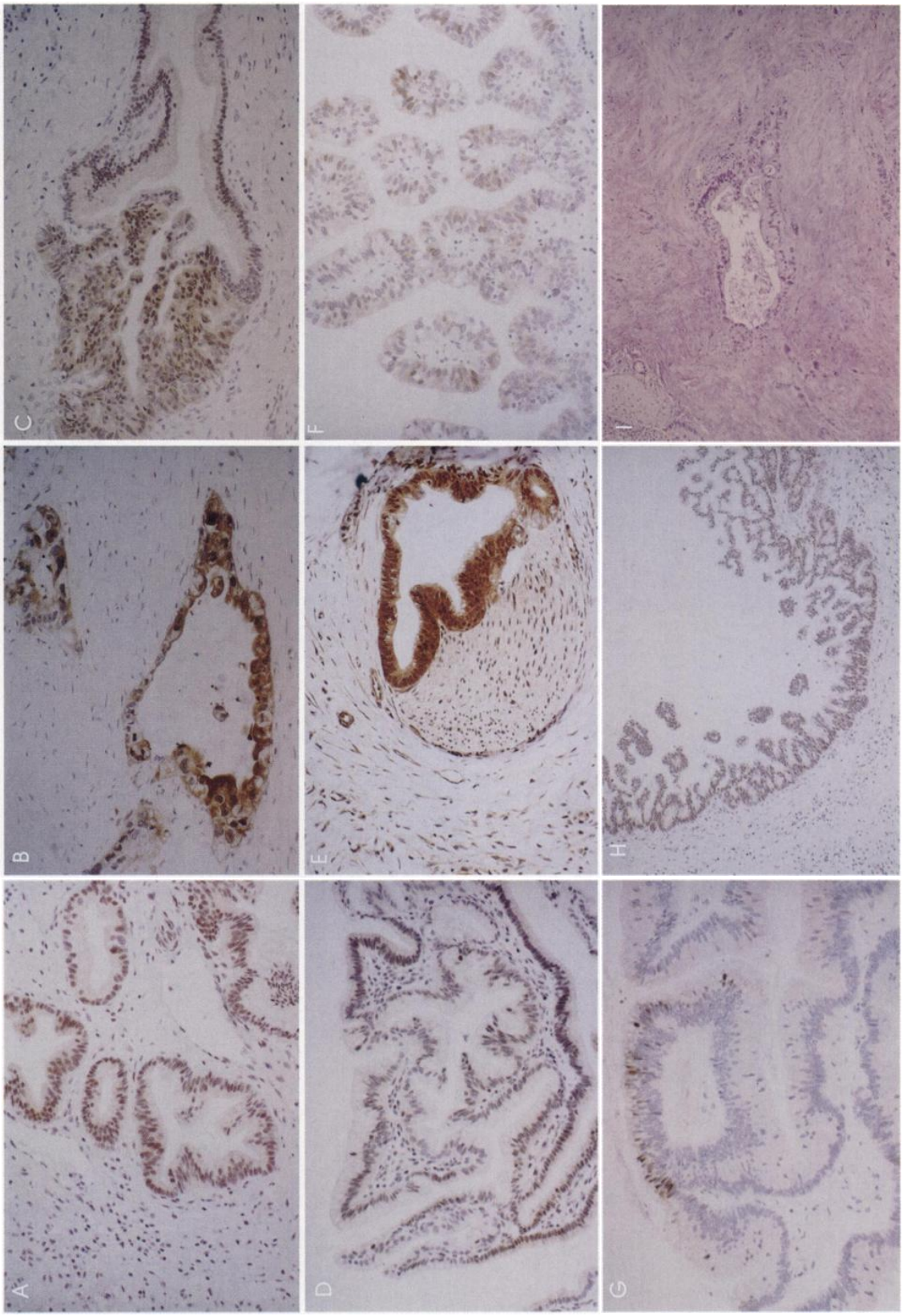

Fig. 1. MGMT immunohistochemistry of human pancreatic cancers. Tissues counterstained with hematoxylin. *A*, infiltrating well-differentiated ductal adenocarcinoma showing diffused positivity for MGMT; magnification, ×200. *B*, infiltrating ductal adenocarcinoma with diffuse strong staining of cells lining the ducts. Pseudostratification and a high degree of dysplasia are evident. Magnification, ×200. *C*, intraductal carcinoma *in situ* adjacent to invasive cancer. Pseudostratified neoplastic cells with marked nuclear atypia show strong immunoreactivity for MGMT. Moderately dysplastic cells show weak or negative reactivity. Magnification, ×200. *D*, papillary hyperplasia with focal high-grade dysplasia. The more dysplastic cells show intense positivity for MGMT as compared to less dysplastic-hyperplastic cells. Magnification, ×200. *E*, perineural invasion by ductal adenocarcinoma of the pancreas. Neoplastic cells lining the gland are positive for MGMT. Magnification, ×250. *F*, intraductal papillary mucinous carcinoma of the main pancreatic duct showing focal positivity for MGMT. Magnification, ×200. *G*, low-grade intraductal papillary carcinoma of the main pancreatic duct showing focal positivity for MGMT. Magnification, ×125. *H*, high-grade *in situ* ductal carcinoma adjacent to invasive cancer showing diffuse and weak activity for MGMT. Micropapillary features are illustrated. Magnification, ×125. *I*, negative control showing lack of reactivity in an infiltrating ductal adenocarcinoma (same as in *B*). Magnification, ×160 (hematoxylin).

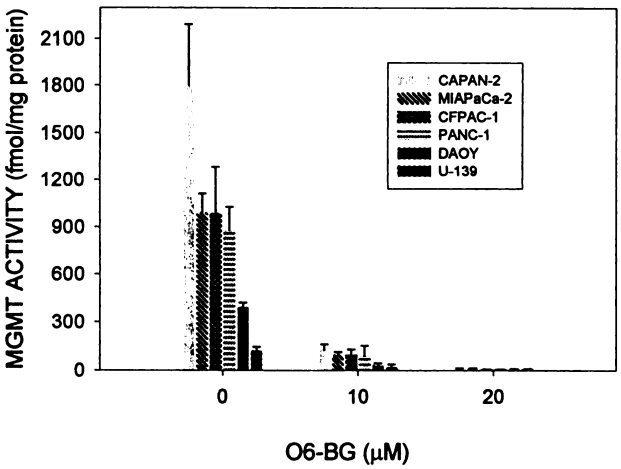

Fig. 2. Depletion of MGMT activity by O^6-BG in four pancreatic adenocarcinoma cell lines. O^6-BG dissolved in medium was added to attached proliferating cells. Cells were harvested 2 h after initiation of treatment and washed extensively with PBS, and the remaining MGMT activity was determined using the biochemical assay.

protein, respectively. MGMT activity, in all of the lines, was completely eliminated with the addition of 10 μM O^6-BG to the culture medium for 4 h or 20 μM of O^6-BG for 2 h. Because O^6-BG concentrations in culture medium did not change significantly with time, frequent changes of O^6-BG-containing medium were not necessary. A depleted MGMT state (<15 fmol/mg protein) was maintained by adding 5 μM of O^6-BG in the medium.

Toxicity of BCNU in the Absence and Presence of O^6-BG. Three pancreatic cell lines tested for toxicity to BCNU were found to be highly resistant compared with Daoy (Fig. 3). In these lines, mitotic activity was somewhat inhibited up to 48 h from treatment without affecting survival following a 45-min incubation with 60 μM BCNU (Table 2), a concentration that is known to eradicate MGMT-deficient cell cultures (43). Severe inhibition of mitosis, however, was evident at BCNU concentrations as low as 40 μM (Table 2), when CFPAC-1 was pretreated with O^6-BG before exposure to BCNU. In addition, treatment of CFPAC-1 with O^6-BG effectively reduced the IC_{50} for BCNU from 200 to less than 40 μM (Fig. 3). Similarly, BCNU inhibited mitosis in PANC-1 (Table 2) and MIAPaCa-2 (data not shown) more effectively when cultures were treated with O^6-BG. Eradication of these two lines (Table 2; Fig. 3) was also more effective when O^6-BG was used to suppress MGMT. Overall, pancreatic tumor cell lines were markedly more resistant to BCNU than was the MGMT-efficient Daoy, which had a moderate MGMT activity. Elimination of MGMT resulted in a similar sensitization of both pancreatic and nonpancreatic cell lines to BCNU, which substantiates the role of MGMT as a common and obviously major mechanism of resistance of a variety of tumor cell lines to the toxic effect of this drug.

Toxicity to MNU in the Absence and Presence of O^6-BG. Of the three pancreatic tumor lines tested, only CFPAC-1 was sensitive to DNA methylation by MNU, with an IC_{50} of approximately 360 μM (Fig. 4). The IC_{50} was reduced to 120 μM when MGMT activity was suppressed with O^6-BG. Complete eradication of the culture was observed following treatment with O^6-BG and 1500 μM MNU or with 2000 μM MNU alone. Daoy was also resistant to MNU and was sensitized by MGMT elimination, as determined by a reduction of the IC_{50} from 500 to 150 μM MNU in the presence of O^6-BG. Complete eradication of this cell line required exposure to 1 mM MNU. Unlike Daoy and CFPAC-1, MIAPaCa-2 and PANC-1 were resistant to MNU, and such resistance was only marginally affected by depletion of MGMT activity. Because cell numbers (shown in Fig. 4) depended not only on cell death, but also on inhibition of cell proliferation via DNA synthesis blocks, we tested the relative contributions of these two factors in the observed reduction of cell numbers in MIAPaCa-2 and PANC-1 cultures treated with MNU. MNU concentrations up to 1000 μM inhibited mitosis in PANC-1 and MIAPaCa-2 cultures at 48 h posttreatment but had no effect on the frequency of cell death. Furthermore, inhibition of DNA synthesis and cell death were not affected by use of O^6-BG. Complete eradication of these two cultures could not be achieved at the concentrations of MNU used, regardless of the presence of the MGMT inhibitor. This suggests that inhibition of cell cycle by MNU (Table 2) is reversible and that the apparent resistance of PANC-1 and MIAPaCa-2 to MNU is due to mechanisms other than the ability to repair O^6-MeG. Due to continued inhibition of MGMT activity by O^6-BG during culture, it is assumed that in these two cultures, cell cycle and DNA synthesis are operational while O^6-MeG lesions in genomic DNA are still present. In contrast to

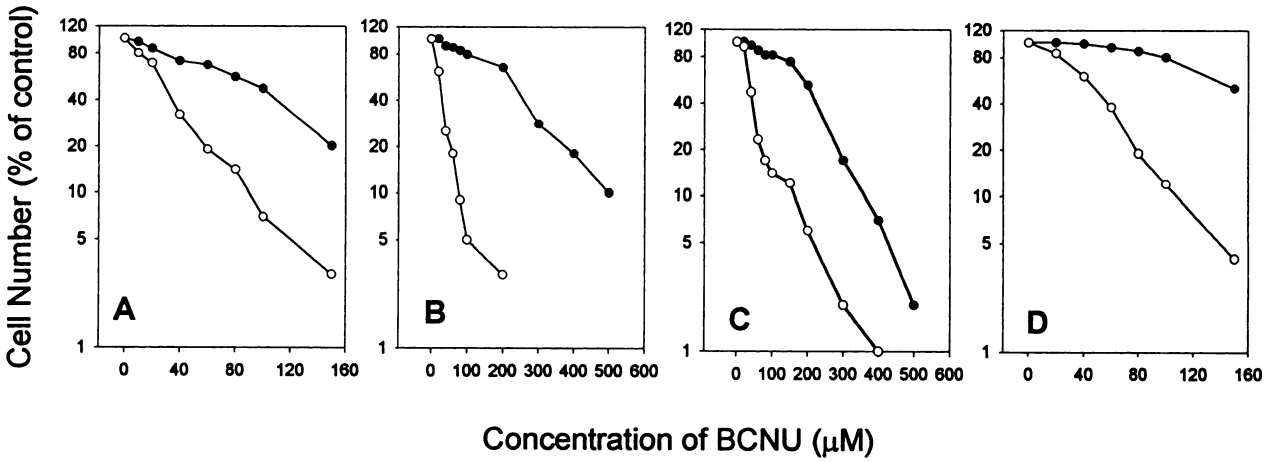

Fig. 3. Potentiation of BCNU toxicity by O^6-BG in Daoy (*A*) and in three pancreatic tumor cell lines, CFPAC-1 (*B*), PANC-1 (*C*), and MIAPaCa-2 (*D*). Exponentially growing cells were treated with 20 μM O^6-BG in full medium for 2 h. Cells were subsequently washed with PBS and treated with varying concentrations of BCNU in PBS for 45 min at 37°C. Cultures were washed again with PBS and supplemented with full medium containing 5 μM O^6-BG. The medium was changed every 48 h for 4 days (before any of the cultures reached confluency). Finally, cells were trypsinized and counted, and survival was determined as a percentage of the control (not treated with BCNU). A parallel experiment was run in which O^6-BG was omitted. Relative survival is shown for cells treated with BCNU alone (●) or with BCNU plus O^6-BG (○).

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