ABSTRACT

Pancreatic carcinoma cells lines are known to have a high incidence of homozygous deletion of the candidate tumor suppressor gene p16 (MTSI/CDKN2), which resides in the chromosome 9p21 region. Here we: (a) examined a series of these cell lines for the incidence of codeletion of genes located near p16, in particular, the gene for the enzyme 5'-deoxy-5'-methylthioadenosine phosphorylase (MTAP) and the genes of the IFNα and β cluster (IFNs); and (b) investigated whether therapeutic strategies could be developed that target malignant cells that have undergone the codeletion of such genes. Five of the eight pancreatic carcinoma cell lines were p16−, MTAP− was codedet in all five cases. Because MTAP phosphorolyses 5'-deoxy-5'-methylthioadenosine (MTA), generated as a byproduct of polyamine synthesis, to the salvageable purine base adenine, loss of this pathway in p16−, MTAP− cells might sensitize these cells to methotrexate (MTX), the mechanism of action of which involves, in part, an inhibition of purine de novo synthesis. MTAP+ normal keratinocytes and pancreatic carcinoma lines had relatively poor sensitivity, in terms of efficacy, to the purine nucleotide-starving actions of MTX. This may be in part due to the MTAP-dependent salvage of adenine moieties from endogenously generated MTA, because the MTAP inhibitor 5'-chloro-5'-deoxyxoforycin A potentiates the antiproliferation of MTX in some of these MTAP+ lines. Also, exogenous MTA (10 μM) reverses the growth-inhibitory actions of MTX in these lines. In contrast, MTAP− cell lines, which cannot recycle purines from endogenous MTA, have a relatively high sensitivity to the antiproliferation of MTX, which is not modulated by 5'-chloro-5'-deoxyxoforycin A or exogenous MTA. Thus the MTAP loss in malignant cells may be an example of gene deletion chemoselectivity, in which genetic deletions that occur as part of the oncogenic process render these cells more sensitive to particular anticancer agents than normal cells, which have not undergone such deletions. We also examined whether the loss of IFN genes sensitize cells to the growth-inhibitory actions of these cytokines. Three of the five p16− cell lines bore homologous deletions of IFNA1 and IFNB1 genes, representing each end of the IFNα, β gene cluster; one cell line bore a codeletion of the IFNA1 gene but retained the IFNB1 locus. Whereas the cell lines that were most sensitive to the growth-inhibitory effects of IFN-β or IFN-α tended to be those with IFN deletions, there were enough exceptions to this pattern to indicate that the IFN genotype does not reliably predict IFN responsiveness.

INTRODUCTION

Loss or inactivation of tumor suppressor genes occurs in many cancers (1). In some cases, both tumor suppressor gene alleles are lost by mechanisms involving genetic deletion, i.e., homozygous deletion. As result of these events, genes that reside nearby but are otherwise unrelated to these tumor suppressor genes can frequently be codeleted. Loss of these “innocent bystander” genes may be significant, because loss of the expression of the proteins encoded by these genes may alter cellular physiology and, as a consequence, the sensitivity of these cells to certain therapeutic agents. Because deletion of these innocent bystander genes occurs in cells undergoing oncogenic transformation but not in normal cells, it may be possible to design therapeutic strategies that take advantage of these selective genetic losses.

A potential example of this involves malignant cells that have undergone genetic deletions in the chromosome 9p21 region. The genes for two candidate tumor suppressor proteins, p16INK4 and p15INK4B, respectively referred to as p16 (MTSI/CDK4/CDKN2) and p15 (MTS2), reside close to one another in this region (2). Both of these proteins act to inhibit the catalytic activity of the cyclin-dependent kinase 4 (or 6)-cyclin D complex, which in its active form phosphorylates the retinoblastoma protein Rb (3, 4); this phosphorylation is thought to be a key step in the progression of cells from a restriction point late in G1, into S-phase (5). Genetic deletion or functional inactivation via mutation of one or both of these cyclin-dependent kinase inhibitors might, in principle, contribute to a loss of the cell’s ability to control cell cycle progression, a hallmark of malignancy (2, 6). Indeed, homozygous deletion or point mutations of the gene for p16 have been shown to occur in a variety of primary malignancies (7–18). Where studied, the p15 gene, which resides approximately 20 kb centromerically from p16 (2, 12), has been shown to be codeleted frequently in malignant cells that bear homozygous deletions of p16 (8, 9, 12, 14, 15). It has been suggested that homozygous deletions within the 9p21 region may be relatively common in particular cancers, because such deletional events could inactivate p16, p15, and possibly other tumor suppressor genes residing in this region simultaneously, resulting in a selective growth advantage (12).

When such deletional events involving multiple loci take place within the 9p21 region, a number of other neighboring genes can be lost coincidently. Examples of this include the gene that encodes the enzyme MTAP3 (MTAP), which is reported to reside less than 100 kb from p16 (19), and the IFN gene cluster, consisting of the IFN-α, β gene (IFNB1) and at least 25 genes and pseudogenes for IFN-α (IFNA) and IFN-ω (20), located approximately 500–1000 kb in the telomeric direction from p16 (19). MTAP is codelleted with a frequency of >85% in cell lines bearing p16 deletions, whereas the IFN gene cluster is deleted in whole or in part in >50% of p16-deficient cell lines (21).

Hypothetically, loss of either MTAP or the IFN genes might render malignant cells with 9p21 defects more sensitive to certain antineoplastic agents than normal cells that have not undergone these genetic deletions. MTAP phosphorolyses the nucleoside MTA, a metabolite of S-adenosylmethionine produced during the synthesis of the poly-
amines spermidine and spermine, to yield 5-methylthioribose-1-phosphate, a methionine precursor, and adenosine (22), which is converted to adenine nucleotides via adenosine phosphoribosyltransferase (EC 2.4.2.7; Refs. 23 and 24). Thus, MTAP plays a critical role in recycling adenine moieties from 5-adenosylmethionine, derived originally from ATP, back to adenine nucleotides pools (25). MTAP-deficient malignant cells, unable to metabolize MTA, simply excrete it (26). Loss of this adenine salvage pathway might sensitize p16

MTAP

cells to the anticancer agent MTX, the mechanism of action of which involves, in part, purine nucleotide starvation via an inhibition of purine de novo synthesis (27). Previous work has demonstrated that one can use exogenous MTA as a purine source to rescue MTAP-containing malignant cells, but not MTAP-deficient malignant lines, from the antipurine actions of MTX and related antifolates (28–30). As for the IFNA and IFNB genes, hypothetically, their loss could render cells more sensitive to the antiproiferative effects of these cytokines (31). This hypothesis is based on the pharmacological phenomenon known as "supersensitivity," in which abrogation of the action of a particular hormone, cytokine, or other chemical signaling molecule, e.g., by the action of a receptor antagonist or, in this case, genetic loss, can lead to a homeostatic up-regulation of the signaling molecule’s receptor levels and/or intracellular signaling machinery (32). If an up-regulation of proteins involved with the cellular signaling response to the IFNs has occurred in p16-deficient malignant cells that bear codeletions of the IFN cluster, such cells might be particularly responsive to the growth-inhibitory actions of IFN-α or -β.

In this report, we examined a series of human pancreatic carcinoma cell lines, a high percentage of which been shown to bear deletions or mutations of p16 (18), for the incidence of codeletion of MTAP, the genes for IFN-α and -β, and other 9p21 markers; we then explored whether chemotherapeutic approaches involving MTX or the IFNs could be developed that are strongly active against the malignant cell types that have lost these loci but less so against normal epithelial cells (low-passage normal human epidermal keratinocytes were used in these studies), which have an unmodified 9p21 region.

MATERIALS AND METHODS

Materials. MTA, MTX, thymidine, and human IFN-β were obtained from Sigma Chemical Co. (St. Louis, MO); IFN-α2b (Hoffman-LaRoche Inc., Nutley, NJ) was obtained from the pharmacy at the University of Massachusetts Medical Center. 8-14C-labeled MTA (55 mCi/mmol) was obtained from Moravek Biochemicals (Brea, CA).

Cell Culture. All pancreatic carcinoma cell lines were obtained from the American Type Culture Collection (Rockville) and cultured according to American Type Culture Collection recommendations. Isolation of genomic DNA and polyadenylated RNA was carried out on ~0.5–2 × 10^9 cells as described previously (21). Normal human epidermal keratinocytes were obtained from Clonetics (San Diego, CA). These cells, obtained at passage 2, were grown in a serum-free KGM medium consisting of modified MCDB 153 medium supplemented with 0.1 ng/ml epidermal growth factor, 5.0 μg/ml insulin, 0.5 μg/ml hydrocortisone, 0.15 mM calcium chloride, and 0.03 mg/ml bovine pituitary extract. All drug sensitivity experiments using keratinocytes were performed on cells from passages 3 or 4.

MTT-based Cell Growth Assay. For studies on the sensitivity of pancreatic carcinoma cell lines to the antipurine actions of methotrexate (MTX) cell cultures growing in FCS-containing medium were washed in PBS, harvested using trypsin-EDTA, and washed twice in the appropriate medium (e.g., DMEM) containing 10% horse serum and 20 μM thymidine. Cells were plated in this thymidine-containing medium (1-ml final volume) in 12-well dishes (20,000 cells/well) containing various concentrations of MTX in the presence or absence of 10 μM MTA (or 25 μM 5'-ClIF). MTT sensitivity experiments involving normal epidermal keratinocytes were performed in thymidine-supplemented, serum-free KGM medium in 24-well dishes (6000 cells/well; 0.5-ml final volume). After 7 days in a humidified 37°C incubator, 200 μl of a solution of MTT (5 mg/ml dissolved in PBS) were added directly to each well, and the plate was incubated an additional 2 h at 37°C. A solution of 0.1 N HCl and 10% Triton X-100 in isopropanol was added to each well (1.2 ml) to dissolve the colored metabolite of MTT, formazan. This solubilization was facilitated by sonicating each well using a Branson 250 sonifier (Danbury, CT). An aliquot (300 μl) of each well was then transferred to individual wells in a 96-well plate, and the absorbance at 570 nm was measured in a Molecular Devices (Sunnyvale, CA) plate reader against a background wavelength of 650 nm. After subtraction of background values (wells containing medium but no cells), the absorbance values were plotted as a percentage of the absorbance value of control wells, in which cells were grown in the absence of MTX. Curve fitting and calculation of IC50 values were performed using the Inplot program (Graphpad, San Diego, CA). Experiments on the sensitivity of the various cell lines to the growth-inhibitory effects of the IFNs were performed in a similar manner, except that the experiments were carried out in thymidine-free medium containing 10% FCS instead of horse serum, or serum-free KGM medium in experiments using normal keratinocytes. Standard curves using cells from each pancreatic carcinoma line demonstrated that the generation of formazan from MTT is linear with cell numbers over a range of 20,000–640,000 cells (up to 1,280,000 cells in the case of the Mia PaCa-2 line); in the case of the normal epidermal keratinocytes, standard curves were linear over a range of 6,000–384,000 cells. These standard curves were used to estimate the number of cell doublings that occurred in the experimental samples.

PCR Analysis of Genomic DNA for 9p21 Markers. Genomic DNA was purified from each of the various cultured cells (0.5–2 × 10^7 cells) as described previously (21). The methods for the PCR-based amplification and analysis of portions of the p16 gene, including p16 Int1/Ex2, p16 Exon 2, and p16 3' (alternatively called CDK4 Int1/Ex2, CDK4 Exon 2, and CDK4 3', respectively), the IFNA1 and IFNB1 genes, and the D9S171 and D9S169 markers have been detailed (21). The TTRP locus was analyzed by the method of Foutain et al. (33). The amplification of a 349-bp segment of exon 2 of the p15 gene (designated p15 Exon 2) was carried out using a modification of a procedure described by Washimi et al. (15). In this case, PCR reaction mixtures (100 μl) contained 0.5 μg genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 0.2 mM deoxynucleotide triphosphates, 2.5 units AmpliTaq DNA polymerase (Perkin Elmer/Cetus, Norwalk, CT), 5% DMSO, and 0.4 μM of each primer (5' primer, 5'-GGCTCTGACACCTGCTCT-3'; 3' primer, 5'-TGGGGTTGGAAAAATGGTAGAAGA-3'). Amplification was performed in a Perkin Elmer/Cetus 480 DNA thermal cycler under the following conditions: 5 min initial denaturation at 94°C, followed by 35 cycles consisting of a denaturation step of 94°C for 45 s, an annealing step of 60°C for 45 s, and an extension step of 72°C for 1 min. PCR products were analyzed using 1.5% agarose gels. The PCR-based genetic deletion results were confirmed using a reverse transcription PCR technique designed to detect transcripts of p16, p15, and the IFNs, as described previously (21).

Assay of MTAP Activity. MTAP activity in cell extracts was examined using a previously described radioassay, which monitors the conversion of 8-14C-labeled MTA to 8-14C-labeled adenosine (21). Protein content was determined using the method of Bradford (34). Cell extracts were determined to be MTAP positive if the activity was greater than 0.01 pmol 8-14C-labeled adenosine formed/min/mg protein, and the activity was linear with time (up to 120 min) and protein content.

RESULTS

Loss of p16 and the Incidence of Codeletion of Genes and Markers within the 9p21 Region of Human Pancreatic Carcinoma Cell Lines. Previous work had demonstrated that the gene encoding p16INK4a was deleted or mutated in a high percentage of human pancreatic cancer cell lines (18); the initial objective of this study was to determine which other genes or markers residing in the chromosome 9p21 region of these cell lines were codeleted along with this candidate tumor suppressor gene. Genomic DNA from eight pancreatic carcinoma cell lines as well as cultures of normal human epidermal keratinocytes were analyzed by PCR or, in the case of MTAP, a functional assay, for the presence of a series of nine markers, which together represent a linkage group of discrete loci across the 9p21 region. IFNβ1, the most telomeric of these markers, represents the
Two analysis deleted, the marker in IFNAJ, and noncoding lies of assayed PANC-1 + Hs766T. The deletion at the centromeric end of the 9p21 region, at a distance estimated to be 5—6 Mb from IFNAJ; D9S169 is believed to reside about 4 centimorgans closer to the centromere than D9S171 (35). The TYPR gene, which has been mapped to the 9p21 region (36), was also examined as part of this PCR-based deleitional analysis to control for the presence of chromosome 9 genomic DNA in each sample.

The results of these deletion studies are presented in Table 1. In this table, + indicates that the expected PCR product for a particular marker was observed, signifying that at least one allele of the gene or marker in question is present in the genomic DNA; − indicates that the PCR product was not observed, indicating a homozygous deletion of that marker within the genome of that cell line. All of the cell lines examined were positive for the TYPR locus (Table 1), indicating that DNA derived from chromosome 9 was present in all the samples.

As shown in Table 1, the Capan-1, BxPC-3, SU86.86, PANC-1, and Mia PaCa-2 pancreatic carcinoma cell lines were found to have homozygous deletions of the p16 gene, confirming the results reported by Caldas et al. (18). In each case, all three regions of the p16 gene that were examined, including a 167-bp segment that spans the 3’ end of intron 1 and 20 bp of exon 2 (p16 In1/Ex2), a 159-bp segment that lies within exon 2 (p16 Exon 2), and a 355-bp portion of the 3’ noncoding end of exon 3 (p16 3’), were deleted in these cell lines. Two of the three pancreatic carcinoma cell lines in which p16 is not deleted, AsPC-1 and HS766T, have been shown to contain mutations within this gene; thus, of the entire series, only the CFPAC-1 line possesses wild-type p16 (18). As expected, neither p16 nor any of the 9p21 markers examined were deleted in low-cell passage (passages 3 and 4), cultured normal human epidermal keratinocytes.

The gene for p15INK4B was codelated in all five pancreatic carcinoma lines bearing homozygous deletions of p16 (Table 1); this result is similar to those obtained in studies involving glioblastomas (12), non-small cell lung carcinoma (14, 15), and leukemias (8, 9), in which the incidences of codelation of the p15 and p16 genes are very high. In addition, none of the five cell lines that lack both p16 and p15 had MTAP activity, indicating that the MTAP locus was also codelated in each case (Table 1). The high incidence of the loss of MTAP in p16-deficient cell lines was predicted from previous work, which suggested that these two genes are codelated with frequencies exceeding 85% (21). Four of the five p16-deficient cell lines displayed a homozygous deletion of IFNA1, whereas homozygous deletion of the IFNB1 gene occurred in three of the five p16-deficient cell lines (Table 1). There was no apparent relationship between cell doubling times and the loss of p16, p15, and/or the IFN genes, the protein products of which, in theory, can act to inhibit cell growth. For instance, the cell-doubling time of normal epidermal keratinocytes (26.7 h), which have no deletions of these genes, is not markedly different than malignant cell lines such as SU86.86 and Mia PaCa-2, which lack p16, p15, IFNA1, and IFNB1 (doubling times, 32.6 and 23.9 h, respectively). In the same vein, Capan-1 cells, which lack p16, p15, and the IFN gene cluster, has a doubling time of 55.6 h which is, in fact, longer than that of the CFPAC-1 line (49.9 h), which bears no such deletions.

Only one cell line, Capan-1, exhibited deletion of the D9S171 marker, suggesting that this line bears a deletion of at least 5—6 Mb within the 9p21 region. None of the cells displayed a loss of the D9S169 marker, the most centromeric of those examined in this study. Taken together, these data support two emerging hypotheses concerning 9p21 deletions: (a) when 9p21 deletions occur, they include the p16 locus virtually always; thus, homozygous deletion of any 9p21 marker is a strong indicator that deletion of p16 has taken place; and (b) the incidence of codelation of other 9p21 loci is roughly inversely proportional to the distance the particular gene maps from p16. For example, the genes that map most closely to p16, e.g., p15 and MTAP, have the highest incidences of codelation, whereas genes in the 9p21 region that map relatively distantly from p16, e.g., IFNB1 and D9S171, have proportionately lower incidences of codelation (21).

MTAP Deletion and Responsiveness to the Antipurine Actions of MTX. One of our hypotheses is that p16-deficient cells with an MTAP locus that is also deleted may be more susceptible to the antipurine actions of antifolates such as MTX than MTAP+ cells, including normal ones. Part of the mechanism of action of MTX involves the inhibition of several folate-requiring steps of purine de novo synthesis, such as glycaminide ribonucleotide transformylase and 5-amino-4-imidazolecarboxamide ribonucleotide transformylase, by polyglutamylated forms of MTX and/or dihydrofolate, the latter accumulating as result of the inhibition of dihydrofolate reductase by MTX (27, 37, 38). As a consequence, nucleotide pools, including purines, may be inadequate to support DNA synthesis during S-phase.

Table 1. Homozygous deletion of 9p21 markers in human pancreatic carcinoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Doubling time (h)</th>
<th>TYPR</th>
<th>IFNB1</th>
<th>IFNA1</th>
<th>MTAP^a</th>
<th>p16 3'</th>
<th>p16 Exon 2</th>
<th>p16 In1/Ex2</th>
<th>D9S171</th>
<th>D9S169</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capan-1</td>
<td>55.6</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>BxPC-3</td>
<td>34.9</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AsPC-1</td>
<td>37.0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SU86.86</td>
<td>32.6</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>HS766T</td>
<td>71.4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PANC-1</td>
<td>31.9</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CFPAC-1</td>
<td>49.9</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mia PaCa-2</td>
<td>23.9</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Normal human epidermal keratinocytes</td>
<td>26.7^d</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

^a MTAP was assessed using the radiochemical assay described in Ref. 21. Enzyme activity, expressed as nmol adenosine formed/min/mg protein is given in parentheses (mean ± SD of at least two determinations). −, no PCR product was observed, indicating a homozygous deletion. All samples were assayed at least twice. TYPR is a marker for 9p23 (36).

^b Two-bp deletion (Ref. 18).
^c Splice site mutation (Ref. 18).
^d Determined by the suppliers.
resulting in cell death (39). The operation of preformed purine salvage pathways, such as that involving MTAP, might act to relieve, in part, the purine nucleotide-limiting effects of MTX. Because MTAP-deficient cells cannot recycle purine moieties derived from S-adenosylmethionine during polyamine synthesis, they might be more sensitive to the purine nucleotide-starving actions of MTX.

To test this, four p16+, MTAP+ cell lines, including normal epidermal keratinocytes, were compared with p16-, MTAP- cell lines in terms of their sensitivity to the antipurine actions of MTX; this was done by performing dose-response curves with MTX on each cell type in media containing 20 μM thymidine, which negates the suppression of thymidine nucleotide synthesis effected by MTX. All of these experiments were performed using media containing horse serum, which lacks MTAP activity (26). Under these conditions, the antipurine-related, growth-inhibitory actions of MTX were relatively weak in the MTAP+ cell lines; in normal keratinocytes, for example, the IC50 value was reasonably high, 4.4 ± 2.2 × 10^-8 M, but the efficacy is poor, with maximal levels of growth inhibition relative to untreated controls of only 60% being achieved (Fig. 1A, ○). In two other MTAP+ cell lines, Hs766T and AsPC-1, both the potency and the efficacy of the antipurine actions of MTX are low (IC50 values, ≥5 × 10^-6 M; maximal inhibition, 45-55% of controls). MTX had a moderate effect on CFPAC-1 cells, with an IC50 of 11.9 ± 3 × 10^-8 M and a maximal growth inhibition approaching 90%. In contrast, the antipurine actions of MTX on the MTAP+ cell lines were relatively strong, with IC50 values of 1.6 ± 0.3, 2.1 ± 0.9, and 11.2 ± 0.8 × 10^-8 M for the relatively fast-growing Mia PaCa-2, SU86.86, and PANc-1 pancreatic carcinoma lines, respectively, and 9.3 × 10^-8 M for the slower-growing Capan-1 line, and high efficacies (maximal levels of growth inhibition, ≥90% relative to untreated controls in each case; Fig. 1B, ○). A fifth MTAP+ cell line, BxPC-3, also displayed high sensitivity, in terms of both potency and efficacy, to the antipurine actions of MTX (Fig. 2, upper right panel). Thus, at least among these pancreatic cell lines, there seemed to be a difference in the efficacy of MTX between cells with MTAP+ and MTAP- genotypes. Coaddition of 10 μM MTA abrogated the antiproliferative effects of MTX in MTAP+ normal keratinocytes, Hs766T, and AsPC-1 cells almost completely and effected a partial reversal in CFPAC-1 cells (Fig. 1A, ○); this occurs because these cells are able to phosphorolyze MTA to adenine, thereby relieving the MTX-mediated blockade of purine de novo synthesis. In marked contrast, exogenous MTA failed to rescue any of the MTAP- cell lines (Fig. 1B, ○). To illustrate the selectivity of this regimen, one can compare the dose-response curves obtained using MTAP+ keratinocytes with those of MTAP- PANC-1 cells; at a MTX concentration of 1 × 10^-6 M in the presence of 10 μM MTX, there is an 86% inhibition of the growth of PANC-1 cells but only a 14% inhibition for that of the keratinocytes. These results are in accord with previous work, which showed that MTA can selectively rescue MTAP+ but not MTAP- malignant cells from the antiproliferative actions of antifolates (28, 30); here, it is demonstrated that this strategy can be used to rescue normal epithelial cells selectively, at least under these specific in vitro conditions.

If, in fact, the functioning of the MTAP-dependent adenine salvage pathway is the reason for the relatively low antipurine-related actions of MTX in MTAP+ cells, then an inhibitor of MTAP, by blocking this adenine salvage mechanism, should potentiate the growth-inhibitory actions of this antifolate in MTAP+ but not MTAP- cell types. Dose-response curves for the antipurine action of MTX were then performed on representative cell lines in the absence or presence of 25 μM 5′-ClF, a competitive inhibitor of MTAP with a Ki value of 0.3 μM (40). At this concentration, which by itself has no effect on the growth of these cell lines, 5′-ClF caused a leftward shift in the dose-response curve of MTX in the MTAP+ cell lines; e.g., the IC50 value of the purine-related antiproliferative actions of MTX is decreased by a factor of >25-fold in Hs766T cells; a similar shift is observed with AsPC-1 cells (Fig. 2). Also, 5′-ClF produced a small (10-20%) but consistently observed increase in the efficacy of the antipurine-based, growth-inhibitory actions of MTX in these MTAP+ cell lines. In contrast, 5′-ClF produces no effect on either the potency or efficacy of the antipurine actions of MTX in two MTAP-deficient cell lines, Mia PaCa-2 and BxPC-3, as predicted. Although the MTAP inhibitor does not increase the efficacy of MTX in MTAP+ cells to the levels observed in MTAP- cell lines, this may be because at this [M]:[K] ratio, a complete blockade of the MTAP reaction is not achieved in intact cells. Overall, the data are consistent with the hypothesis that the
endogenous recycling of adenine from MTA in part antagonizes the purine nucleotide-starving actions of MTX.

**Loss of the IFN Genes and Responsiveness to IFNs.** The deletion of the IFN cluster represents another potentially exploitable difference between malignant cells with 9p21 defects and normal cells. We hypothesized that cells that have undergone a complete or partial deletion of the interferon cluster might respond to this loss with a homeostatic up-regulation of IFN-α and -β receptors and/or intracellular signaling components, which, in turn, might make the cell unusually sensitive to the growth-inhibitory actions of IFN-β or -α. In the case of IFN-β, cell lines that bear homozygous deletions of IFN genes tended to be more responsive, in terms of IC₅₀ values, to this
cytokine than IFN* cell lines (Fig. 3). For example, two p16*, MTAP* cell lines in which the IFN gene cluster is codeleted, Capan-1 and Mia PaCa-2, are relatively sensitive to IFN-β (IC50 values, 31 ± 9 and 41 ± 3 IU/ml, respectively; Fig. 3, upper left panel). Also, BxPC-3, which harbors a partial deletion of the IFN gene cluster ([IFNA1*, IFNB1*]), is highly sensitive to the antiproliferative actions of IFN-β (IC50, 6.8 ± 0.7 IU/ml; Fig. 3, upper right panel). These IC50 values are markedly lower than those of cell types that are p16*, MTAP*, IFNA1*, IFNB1*, including normal keratinocytes, CFPAC-1, AsPC-1, and HS766T (IC50 values, 305 ± 26, 934 ± 110, >4000, and 780 ± 150 IU/ml, respectively; Fig. 3, lower left panel) or p16**, MTAP**, IFNA1**, IFNB1**, i.e., PANC-1 (IC50 value, >4000 IU/ml; Fig. 3, lower right panel). Contrary to this trend, a third pancreatic carcinoma line with the p16**, MTAP**, IFNA1**, IFNB1** genotype, SU86.86, had a relatively high IC50 value for IFN-β, 1408 ± 86 IU/ml, which falls in the range of the IC50 values of IFN* cells; thus, the loss of the IFN gene cluster does not necessarily ensure a high degree of sensitivity to the antiproliferative actions of IFNs.

The responsiveness of individual cell lines to one of the clinically active forms of IFN-α, IFN-α2b, was also examined (Fig. 4). In general, the observed IC50 values were considerably higher than those for IFN-β and did not correlate well with IFN genotype. Taking all the IFN data together, although some trends were observed, the absence or presence of these IFN genes was not a reliable predictor of responsiveness of individual cell lines to the IFNs.

DISCUSSION

In this report, we present evidence that the loss of the gene encoding MTAP (MTAP), which occurs frequently in conjunction with the deletion of the putative tumor suppressor genes p16 and p15 in subsets of malignant cells, renders these cells more sensitive to the anticancer agent MTX than normal cells, which have not undergone these deletions, and hence is an example of gene deletion chemoselectivity. The primary mechanism of loss of MTAP activity in tumors seems to be homozygous deletion, based on genetic deletion mapping studies (Table 1; Refs. 19 and 21). Our original rationale for undertaking this study was that, because the loss of MTAP would disrupt the pathway by which the adenine moiety of MTA is recycled back to purine nucleotide pools, malignant cells lacking this enzyme might be more dependent on purine de novo biosynthesis and, hence, more sensitive to the effects of antifolates such as MTX, the action of which involves in part an inhibition of the purine de novo pathway. It is noteworthy that some MTAP− leukemias have severalfold higher rates of purine de novo biosynthesis than do MTAP+ leukemias (41). Indeed, when the antipurine effects of MTX were studied using media containing thymidine to abrogate the antipyrimidine actions of MTX, this antifolate was markedly more efficacious against the five MTAP−pancreatic carcinoma lines than against the MTAP+ cell lines, including normal keratinocytes (Figs. 1 and 2). In some of the MTAP+ cell lines, the purine-related, growth-inhibitory effect of MTX seemed to plateau (at concentrations in the range of 10−6 M; see Figs. 1 and 2). One can hypothesize that the purine de novo pathway is inhibited completely at this point, but these cells are able to survive in part because of efficient recycling of preformed purines. However, if operation of the MTAP-dependent purine salvage pathway was solely responsible for antagonizing the antipurine actions of MTX, then an MTAP inhibitor ought to enhance the responsiveness of MTAP+ cells to the antipurine actions of MTX. A concentration (25 μM) of the MTAP inhibitor 5′-CIF, which by itself does not produce antiproliferative effects, was found to cause a leftward shift in the dose-response curve of MTX for several of the MTAP+ pancreatic carcinomas but only slight increases in the efficacy of MTX. Part of the reason for this may be the fact that at this concentration, MTAP inhibitors may
not effect a full blockade of MTAP activity in intact cells, but one also must consider the possibility that MTAP− cells undergo other, as-yet undefined, adaptations to MTAP deficiency, perhaps involving changes in nucleotide and/or folate metabolism, which in some way sensitizes them to antifolates. For example, it may be relevant that MTA is a source of formate (42), which can be used readily in folate metabolism, including the de novo purine pathway; this formate source would not be available to MTAP− cells, perhaps causing a perturbation of their folate metabolism. Codeletion of other gene(s) in the 9p21 region, the losses of which sensitize cells to antifolates, cannot be ruled out at this time. Whatever the precise mechanism, these finding suggest that the presence of MTAP in cells predicts a more refractory response to the antipurine actions of MTX, especially in the higher dose range, and that the loss of MTAP may be one factor in determining why MTX can be relatively tumor selective in some circumstances. Obviously, the ultimate test of this in vitro observation would be to determine in clinical situations whether MTAP− tumors are, in fact, more responsive to antifolates with antipurine activity than are MTAP+ tumors. The presence or absence of MTAP within a tumor might be a crucial parameter in predicting the effectiveness of an antifolate such as 5,10-dideazatetrahydrofolate, a selective inhibitor of purine de novo synthesis (43), which is currently undergoing clinical trials (44). The recent isolation of MTAP CDNA (19) opens the way for the development of in situ hybridization probes for making such a determination on tumor samples. Interestingly, a MTAP− non-small cell lung carcinoma cell line has been shown to be somewhat more sensitive to this antifolate than a MTAP+ line (30). The hypothesis that endogenous operation of the MTAP-dependent purine salvage pathway may antagonize the antipurine actions of MTX is also intriguing from the standpoint of drug development, because two of the early model systems for screening the activity of anticancer drugs, the L1210 and P388 murine leukemias, are MTAP deficient (45); this may explain, in part, their high degree of sensitivity to antifolates.

The idea of taking chemotherapeutic advantage of MTAP deficiency in subsets of malignant cells was first proposed by Kamatani et al. in 1981 (28); these investigators demonstrated that one can use exogenous MTA as a purine source to selectively rescue MTAP-containing cells but not MTAP-deficient cells from the actions of the purine de novo inhibitors azaserine or MTX (with thymidine coadded). However, several difficulties might limit the effectiveness of this approach in vivo: first, the presence of MTAP in serum (46) might catalyze the cleavage of MTA to adenine extracellularly, inadvertently rescuing the MTAP-deficient malignant cell from the antipurine effects of a drug such as MTX. Metabolic cooperativity also might take place, in which normal, MTAP-positive cells provide purines to the MTAP-deficient tumor cells, especially when an exogenous purine source such as MTA is administered. Second, in our experience, MTA analogues that behave as alternative substrates of MTAP have very short biological T1/2 values (on the order of several minutes; Ref. 47); thus, there may be pharmacokinetic difficulties in administering MTA as a rescuing agent. These problems emphasize the importance of the current findings, which suggest that, at least under defined conditions, MTX displays more efficacy against MTAP− malignant cells than MTAP+ cells, including normal epithelial cells, even in the absence of MTA coaddition.

As part of these studies, we asked whether the loss of these IFN genes might sensitize cells to the antiproliferative actions of these cytokines, perhaps due to a supersensitivity effect. Whereas the cell lines that were most sensitive to the growth-inhibitory effects of IFN-β or -α2, tended to be those with IFN deletions, there were enough exceptions to this trend to warrant the conclusion that the IFN genotype does not predict responsiveness to the IFNs reliably, at least in the pancreatic carcinoma cell lines. Similar conclusions were reached in a study on the sensitivity of glioma cell lines that either have or lack the 9p21 IFN gene cluster to the growth-inhibitory actions of IFN-α and -β (48). In fact, the biological significance, if any, of homozygous deletion of the 9p21 IFN gene cluster remains unclear. To date, we have been unable to correlate the loss of these genes with changes in doubling times or the ability to respond to the IFN-inducing, double-stranded RNA species polyinosinic-polycytidylic acid (21). One inference that can be made is that the growth-inhibitory actions of these IFNs are not mediated through p16INK4a or p15INK4b, if that were the case, p16−, p15− cell lines would have been uniformly refractory to the IFNs.

One of the general themes presented here is that malignancies of the same histological type do not necessarily have the same genotype and, therefore, may not respond to antineoplastic agents in a similar manner. Indeed, of the eight pancreatic carcinoma cell lines in the present study, five distinct genotypes were identified, and that was based on analysis of only several markers within the 9p21 region. As discussed above, those cell types with the MTAP+ genotype predictably responded to MTX differently than did those with the MTAP− genotype. The concept of gene deletion chemoselectivity suggests that by defining genotypes of a tumor sample, ideally one might be able to choose a chemotherapeutic regimen directed against the particular tumor that has a relatively favorable therapeutic index. Whereas these principles have an appealing logic and can operate under controlled in vitro conditions, it may not be easy to apply them to practical clinical situations. Unfavorable drug distribution or pharmacokinetics, metabolic cooperativity, low growth fractions, and the tendency to develop drug resistance, among many other parameters, may limit the effectiveness of a particular drug, which, in theory, should display high selectivity and sensitivity based on the tumor’s genotype. Factors such as these may explain why, although 9p21 deletions involving p16 (and probably MTAP) are common among primary pancreatic carcinomas (18) and our data indicate that MTX is especially active against MTAP− pancreatic carcinoma cell lines, antifolates generally do not produce remissions in this malignancy (49). If analysis of the tumor’s MTAP genotype is to be of any predictive value in the clinic, i.e., to distinguish potential responders from nonresponders, it would be of value most probably in cancers in which MTX has proven activity, e.g., acute lymphoblastic leukemia in children.

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