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Gene deletion chemoselectivity: codeletion of the genes for p16(INK4), methylthioadenosine phosphorylase, and the alpha- and beta-interferons in human pancreatic cell carcinoma lines and its implications for chemotherapy

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Et al.

Gene Deletion Chemoselectivity: Codeletion of the Genes for p16^{INK4}, Methylthioadenosine Phosphorylase, and the α- and β-Interferons in Human Pancreatic Cell Carcinoma Lines and Its Implications for Chemotherapy[1]

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ABSTRACT

Pancreatic carcinoma cells lines are known to have a high incidence of homozygous deletion of the candidate tumor suppressor gene *p16* (*MTS1/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 IFN-α 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 codeleted in all five cases. Because MTAP phosphorolyzes 5′-deoxy-5′-methylthioadenosine (MTA), generated as a by-product 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′-deoxyformycin A potentiates the antipurine actions 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 antipurine actions of MTX, which is not modulated by 5′-chloro-5′-deoxyformycin 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 homozygous deletions of *IFNA1* and *IFNB1* genes, representing each end of the IFN-α,-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_{2b} 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, p16^{INK4} and p15^{INK4B}, respectively referred to as *p16* (*MTS1/CDK4I/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 G$_1$ 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 coincidentally. Examples of this include the gene that encodes the enzyme MTAP[3] (*MTAP*), which is reported to reside less than 100 kb from *p16* (19), and the IFN gene cluster, consisting of the IFN-β_1 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 codeleted 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 phosphorolyzes the nucleoside MTA, a metabolite of *S*-adenosylmethionine produced during the synthesis of the poly-

[3] The abbreviations used are: MTAP, 5′-deoxy-5′-methylthioadenosine phosphorylase; 5′-ClF, 5′-chloro-5′-deoxyformycin A; IU, international unit; KGM, keratinocyte growth medium; MTA, 5′-deoxy5′-methylthioadenosine; MTX, methotrexate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IC$_{50}$, 50% inhibitory concentration.

amines spermidine and spermine, to yield 5-methylthioribose-1-phosphate, a methionine precursor, and adenine (22), which is converted to adenine nucleotides via adenine phosphoribosyltransferase (EC 2.4.2.7; Refs. 23 and 24). Thus, MTAP plays a critical role in recycling adenine moieties from *S*-adenosylmethionine, derived originally from ATP, back to adenine nucleotide 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 antiproliferative 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-α₂ᵦ (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 × 10⁷ 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 protein/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'-ClF). MTX 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 substraction 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 × 10⁷ 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 *CDK41 Int1/Ex2*, *CDK41 Exon 2*, and *CDK41 3'*, respectively), the *IFNA1* and *IFNB1* genes, and the *D9S171* and *D9S169* markers have been detailed (21). The *TYRP* locus was analyzed by the method of Fountain *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'-GGCTCTGACCACTCTGCTCT-3'; 3' primer, 5'-TGGGGGTGGGAAATTGGGTAAGAA-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 adenine (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 nmol 8-¹⁴C-labeled adenine 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 p16^INK4 was deleted or mutated in a high percentage of human pancreatic 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. *IFNB1*, the most telomeric of these markers, represents the

distal end of the IFN cluster and maps ~1000 kb from the *p16* locus; *IFNA1*, near the proximal end of the IFN cluster, maps >500 kb from *p16*; *MTAP* maps to a locus <100 kb in the telomeric direction from *p16* (19). The gene encoding p15^{INK4} (*p15/MTS2*) resides 15–20 kb from *p16*, in the centromeric direction (2, 12). The microsatellite marker *DS9171* is located at the more centromeric end of the 9p21 region, at a distance estimated to be 5–6 Mb from *IFNA1*; *DS9169* is believed to reside about 4 centiMorgans closer to the centromere than *D9S171* (35). The *TYRP* gene, which has been mapped to the 9p23 region (36), was also examined as part of this PCR-based deletional 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 *TYRP* 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 Int1/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 p15^{INK4B} was codeleted in all five pancreatic carcinoma lines bearing homozygous deletions of *p16* (Table 1); this result is similar to those obtained in studies involving gliomas (12), non-small cell lung carcinoma (14, 15), and leukemias (8, 9), in which the incidences of codeletion 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 codeleted 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 codeleted 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 SU.86.86 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 (55.6 h) that 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 codeletion 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 codeletion, whereas genes in the 9p21 region that map relatively distantly from *p16*, *e.g.*, *IFNB1* and *D9S171*, have proportionately lower incidences of codeletion (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 glycinamide 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*

All markers except MTAP were assessed by PCR amplification of genomic DNA, as described in "Materials and Methods." +, the expected PCR product for the particular marker was observed, demonstrating the presence of at least one copy of the DNA segment in question; −, no PCR product was observed, indicating a homozygous deletion. All samples were assayed at least twice. *TYRP* is a marker for 9p23 (36).

Cell line	Doubling time (h)	TYRP	IFNB1	IFNA1	MTAP[a]	p16 3′	p16 Exon 2	p16 Int1/Ex2	p15 Exon 2	D9S171	D9S169
Capan-1	55.6	+	−	−	−	−	−	−	−	−	+
BxPC-3	34.9	+	+	−	−	−	−	−	−	+	+
AsPC-1	37.0	+	+	+	+ (0.46 ± 0.05)	+	+[b]	+	+	+	+
SU.86.86	32.6	+	−	−	−	−	−	−	−	+	+
Hs766T	71.4	+	+	+	+ (1.05 ± 0.29)	+	+[c]	+	+	+	+
PANC-1	31.9	+	+	+	−	−	−	−	−	+	+
CFPAC-1	49.9	+	+	+	+ (0.46 ± 0.08)	+	+	+	+	+	+
Mia PaCa-2	23.9	+	−	−	−	−	−	−	−	+	+
Normal human epidermal keratinocytes	26.7[d]	+	+	+	+ (0.45 ± 0.05)	+	+	+	+	+	+

[a] MTAP was assessed using the radiochemical assay described in Ref. 21. Enzyme activity, expressed as nmol adenine formed/min/mg protein is given in parentheses (mean ± SD of at least two determinations). −, the MTAP activity in the cell extract was <0.01 nmol adenine formed/min/mg protein.

[b] Two-bp deletion (Ref. 18).

[c] Splice site mutation (Ref. 18).

[d] Determined by the suppliers.
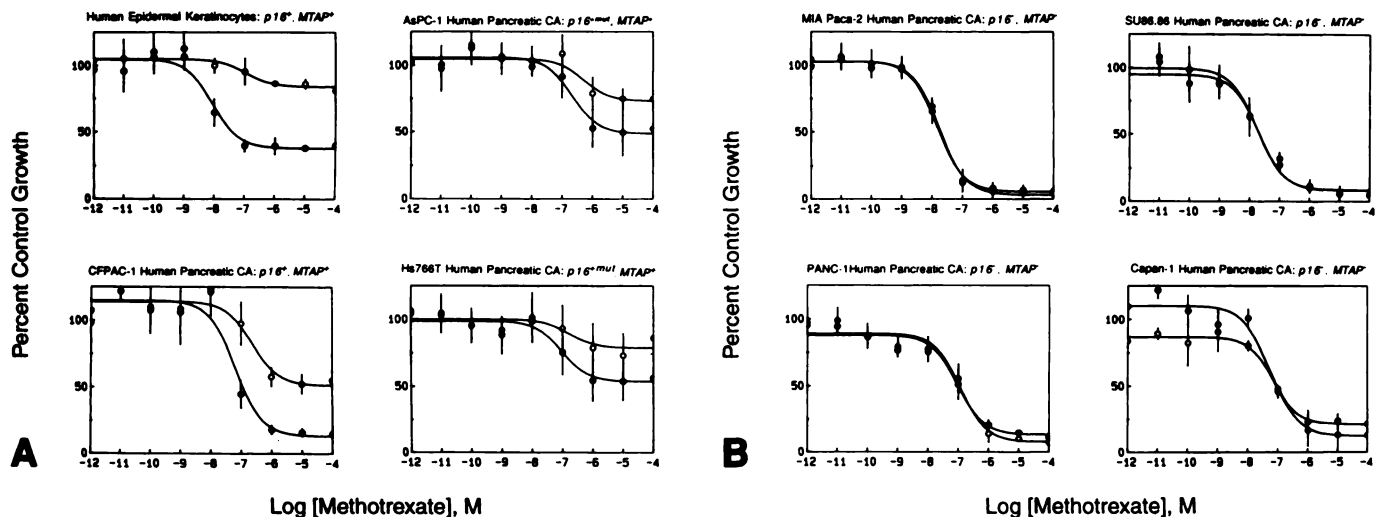
Log [Methotrexate], M

Log [Methotrexate], M

Fig. 1. Dose-response curves for the antipurine-related, growth-inhibitory effects of MTX (●) or MTX plus 10 μM MTA (○) in *MTAP*-positive (*A*) or *MTAP*-negative (*B*) cell lines. Experimental conditions are described in "Materials and Methods." All experiments were carried out in the presence of 20 μM thymidine to eliminate the pyrimidine-depleting effects of MTX. Unless otherwise noted, wells were inoculated with 20,000 cells. Each data *point* represents the mean ± SD of four to six independent samples obtained in two or three experiments. Control (no addition of MTX or MTA) cell doublings: *A*, epidermal keratinocytes (initial cell number/well, 6,000) 4.43; AsPC-1, 4.63; CFPAC-1, 4.20; and Hs766T, 2.16; *B*, Mia PaCa-2, 5.93; SU86.86, 3.49; PANC-1, 4.20; and Capan-1, 4.19. Nonlinear regression curve fitting was carried out using the Inplot program.

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 low, 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. 1*A*, ●). 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*⁻ cells 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. 1*B*, ●). 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. 1*A*, ○); 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. 1*B*, ○). 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 MTA, 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 K₁ 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 [I]:K₁ ratio, a complete blockade of the MTAP reaction is not achieved in intact cells.[4] Overall, the data are consistent with the hypothesis that the

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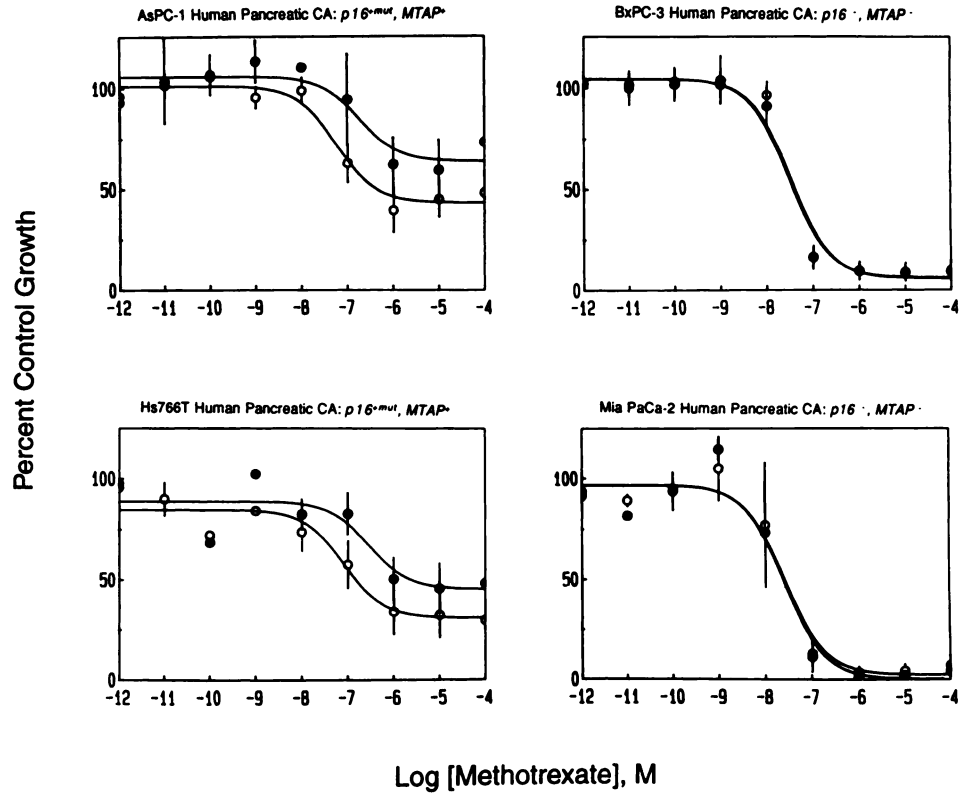[4] T. M. Savarese, unpublished data.

Fig. 2. Effect of the MTAP inhibitor 5'-ClF on the growth-inhibitory antipurine actions of MTX in *MTAP*-positive (*left panels*) and -negative (*right panels*) cell lines. ●, MTX alone; ○, MTX in the presence of 25 μM 5'-ClF. Experimental conditions are described in "Materials and Methods" and the legend of Fig. 1. Values represent the mean ± SD of two to six independent samples. The Inplot program was used to perform nonlinear regression curve fitting.

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 inteferon 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 IC_{50} values, to this
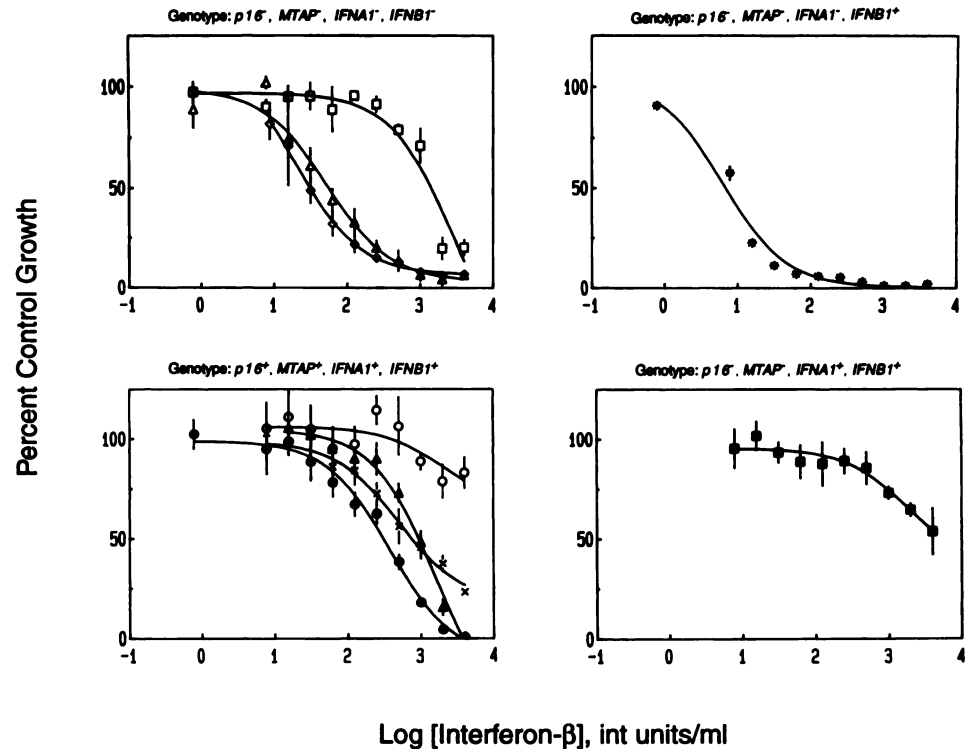

Fig. 3. Dose-response curves for the growth-inhibitory effect of human IFN-β on cell lines of varying 9p21 genotypes in which the *IFNA1* and *IFNB1* genes are present or deleted. Experimental conditions are described in "Materials and Methods." *Upper left panel*, cell lines with the genotype *p16⁻*, *MTAP⁻*, *IFNA1⁻*, *IFNB1⁻*: Mia PaCa-2 (△), control cell doublings, 4.87; Capan-1 (◇), control cell doublings, 6.09; and SU86.86 (□), control cell doublings, 2.83. *Upper right panel*, cell lines with the genotype *p16⁻*, *MTAP⁻*, *IFNA1⁻*, *IFNB1⁺*: BxPC-3 (*), control cell doublings, 4.93. *Lower left panel*, cell lines with the genotype *p16⁺*, *MTAP⁺*, *IFNA1⁺*, *IFNB1⁺*: normal human keratinocytes (●), control cell doublings, 5.31; AsPC-1 (○), control cell doublings, 4.87; CFPAC-1 (▲), control cell doublings, 5.04; Hs766T (X), control cell doublings, 2.33. *Lower right panel*, cell lines with the genotype *p16⁻*, *MTAP⁻*, *IFNA1⁺*, *IFNB1⁺*: PANC-1 (■), control cell doublings, 4.04. Data *points* and error *bars* represent the mean ± SD of four to six determinations performed in two or three independent experiments. Nonlinear regression curve fitting was carried out using the Inplot program.

Genotype: *p16⁻, MTAP⁻, IFNA1⁻, IFNB1⁻*

Genotype: *p16⁻, MTAP⁻, IFNA1⁻, IFNB1⁺*

Genotype: *p16⁺, MTAP⁺, IFNA1⁺, IFNB1⁺*

Genotype: *p16⁻, MTAP⁻, IFNA1⁺, IFNB1⁺*

Fig. 4. Dose-response curves for the growth-inhibitory effect of human IFN-α₂ᵦ on cell lines of varying 9p21 genotypes in which the *IFNA1* and *IFNB1* genes are present or deleted. Experimental conditions are described in "Materials and Methods." *Upper left panel,* cell lines with the genotype *p16⁻, MTAP⁻, IFNA1⁻, IFNB1⁻*: Mia PaCa-2 (△), control cell doublings, 5.77; Capan-1 (◇), control cell doublings, 4.01; and SU86.86 (□), control cell doublings, 2.49. *Upper right panel,* cell lines with the genotype *p16⁻, MTAP⁻, IFNA1⁻, IFNB1⁺*: BxPC-3 (*), control cell doublings, 4.81. *Lower left panel,* cell lines with the genotype *p16⁺, MTAP⁺, IFNA1⁺, IFNB1⁺*: normal human keratinocytes (●), control cell doublings, 5.27; AsPC-1 (○), control cell doublings, 5.77; CFPAC-1 (▲), control cell doublings, 4.72; and Hs766T (X), control cell doublings, 2.37. *Lower right panel,* cell lines with the genotype *p16⁻, MTAP⁻, IFNA1⁺, IFNB1⁺*: PANC-1 (■), control cell doublings, 3.5. Data *points* and error *bars* represent the mean ± SD of four to six determinations performed in two or three independent experiments. Nonlinear regression curve fitting was carried out using the Inplot program.

Log [Interferon-α₂ᵦ], int units/ml
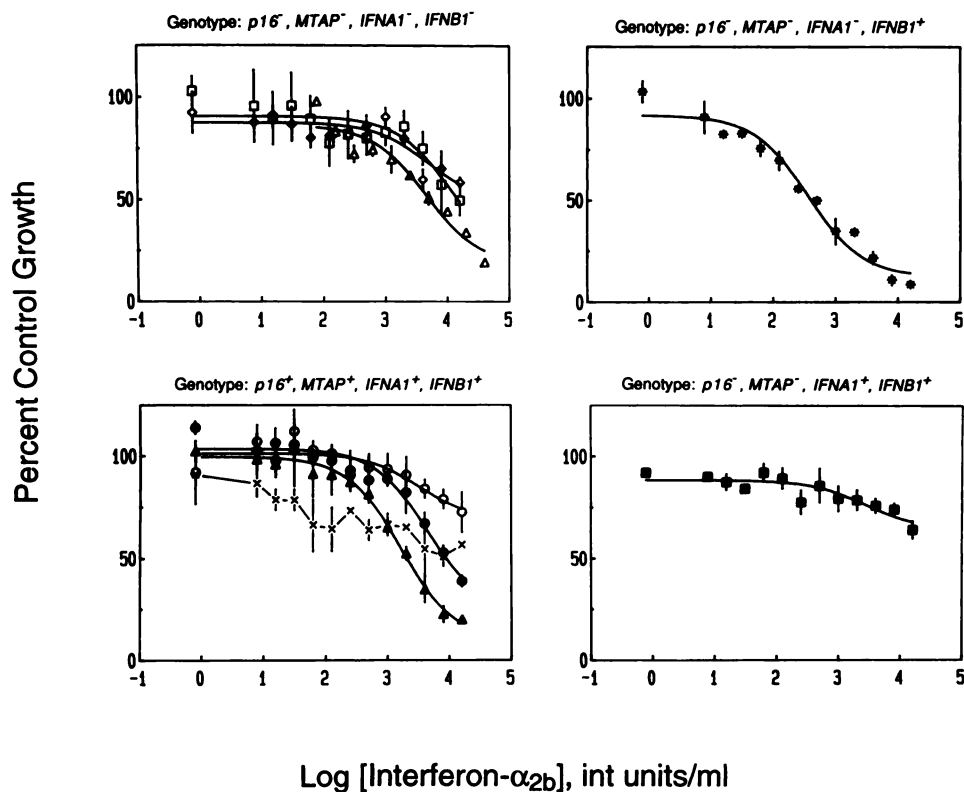
cytokine than *IFN⁺* cell lines (Fig. 3). For example, two *p16⁻, MTAP⁻* cell lines in which the IFN gene cluster is codeleted, Capan-1 and Mia PaCa-2, are relatively sensitive to IFN-β (IC₅₀ values, 31 ± 9 and 41 ± 3 IU/ml, respectively; Fig. 3, *upper left panel*). Also, BxPC-3, which harbors a partial 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-α₂ᵦ, 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'-ClF, 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,[5] 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 $t_{1/2}$ 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 -α₂ᵦ 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 $p16^{INK4}$ or $p15^{INK4B}$; 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.

ACKNOWLEDGMENTS

REFERENCES

1. Weinberg, R. A. Tumor suppressor genes. Science (Washington DC), *254:* 1138–1146, 1991.
2. Kamb, A. Gruis, N. A., Weaver-Fledhaus, J., Liu, Q., Harshman, K., Tavtigian, S. V., Stockert, E., Day, R. S. III, Johnson, B. E., and Skolnick, M. H. A cell cycle regulator potentially involved in the genesis of many tumor types. Science (Washington DC), *264:* 436–440, 1994.
3. Serrano, M., Hannon, G. J., and Beach, D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature (Lond.), *366:* 704–707, 1993.
4. Hannon, G. J., and Beach, D. p15^INK4B is a potential effector of TGF-β-induced cell cycle arrest. Nature (Lond.), *371:* 257–261, 1994.
5. Sherr, C. J. G₁ phase progression: cyclins on cue. Cell, *79:* 551–555, 1994.
6. Nobori, T., Mirua, K., Wu, D. J., Lois, A., Takabayashi, K., and Carson, D. A. Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature (Lond.), *368:* 753–756, 1994.

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[5] T. M. Savarese, unpublished data.

7. Ogawa, S., Hirano, N., Sato, N., Takahashi, T., Hangaishi, A., Tanaka, K., Kurokawa, M., Tanaka, T., Mitani, K., Yazaki, Y., and Hirai, H. Homozygous loss of the cyclin-dependent kinase 4-inhibitor (*p16*) gene in human leukemias. Blood, *84:* 2431–2435, 1994.

8. Hebert, J., Cayuela, J. M., Berkeley, J., and Sigaux, F. Candidate tumor-suppressor genes *MTS-1* (*p16*^{INK4A}) and *MTS-2* (*p15*^{INK4B}) display frequent homozygous deletions in primary cells from T- but not from B-cell lineage acute lymphoblastic leukemias. Blood, *84:* 4038–4044, 1994.

9. Otsuki, T., Clark, H. M., Wellmann, A., Jaffe, E. S., and Raffeld, M. Involvement of *CDKN2* (*p16*^{INK4A}/*MTS1*) and *p15*^{INK4B}/*MTS2* in human leukemias and lymphomas. Cancer Res., *55:* 1436–1440, 1995.

10. Hussussian, C. J., Struewing, J. P., Goldstein, A. M., Higgins, P. A. T., Ally, D. S., Sheahan, M. D., Clark, W. H., Jr., Tucker, M. A., and Dracopoli, N. C. Germline *p16* mutations in familial melanoma. Nat. Genet., *8:* 15–21, 1994.

11. Reed, J. A., Loganzo, F., Jr., Shea, C. R., Walker, G. J., Flores, J. F., Glendening, J. M., Bogdany, J. K., Shiel, M. J., Haluska, F. G., Fountain, J. W. F., and Albino, A. P. Loss of expression of the *p16*/cyclin-dependent kinase inhibitor 2 tumor suppressor gene in melanocytic lesions correlates with invasive stage of tumor progression. Cancer Res., *55:* 2713–2718, 1995.

12. Jen, J., Harper, J. W., Bigner, S., Bigner, D. D., Papadopoulos, N., Markowitz, S., Willson, J. K. V., Kinzler, K. W., and Vogelstein, B. Deletion of *p16* and *p15* genes in brain tumors. Cancer Res., *54:* 6353–6358, 1994.

13. Shapiro, G. I., Edwards, C. D., Kobzik, L., Godleski, J., Richards, W., Sugarbaker, D. J., and Rollins, B. J. Reciprocal Rb inactivation and p16^{INK4} expression in primary lung cancers and cell lines. Cancer Res., *55:* 505–509, 1995.

14. Xiao, S., Li, D., Corson, J. M., Vijg, J., and Fletcher, J. A. Codeletion of *p15* and *p16* genes in primary non-small cell lung carcinoma. Cancer Res., *55:* 2968–2971, 1995.

15. Washimi, O., Nagatake, M., Osada, H., Ueda, R., Koshikawa, T., Seki, T., Takahashi, T., and Takahashi, T. *In vivo* occurrence of *p16* (*MTS1*) and *p15* (*MTS2*) alterations preferentially in nonsmall cell lung cancers. Cancer Res., *55:* 514–517, 1995.

16. Mori, T., Miura, K., Aoki, T., Nishihira, T., Mori, S., and Nakamura, Y. Frequent somatic mutation of the *MTS1/CDK41* (multiple tumor suppressor/cyclin-dependent kinase 4 inhibitor) gene in esophageal squamous cell carcinoma. Cancer Res., *54:* 3396–3397, 1994.

17. Cheng, J. Q., Jhanwar, S. C., Klein, W. M., Bell, D. W., Lee, W-C., Altomare, D. A., Nobori, T., Olopade, O. I., Buckler, A. J., and Testa, J. R. *p16* alterations and deletion mapping of 9p21-p22 in malignant mesotheliomas. Cancer Res., *54:* 5547–5551, 1994.

18. Caldas, C., Hahn, S. A., daCosta, L. T., Redston, M. S., Schutte, M., Seymour, A. B., Weinstein, C. L., Hruban, R. H., Yeo, C. J., and Kern, S. E. Frequent somatic mutations and homozygous deletions of the *p16* (*MTS1*) gene in pancreatic adeno-carcinomas. Nat. Genet., *8:* 27–32, 1994.

19. Olopade, O. I., Pomykala, H. M., Hagos, F., Sveen, L. W., Espinosa, R. III, Dreyling, M. H., Gursky, S., Stadler, W. M., LeBeau, M. M., and Bohlander, S. K. Construction of a 2.8 megabase yeast artificial chromosome contig and cloning of the human methylthioadenosine phosphorylase gene from the tumor suppressor region on 9p21. Proc. Natl. Acad. Sci. USA, *92:* 6489–6493, 1995.

20. Diaz, M. O., Ziemin, S., LeBeau, M. M., Pitha, P., Smith, S. D., Chilcote, R. R., and Rowley, J. D. Homozygous deletion of the α- and β-interferon genes in human leukemia and derived cell lines. Proc. Natl. Acad. Sci. USA, *85:* 5259–5263, 1988.

21. Zhang, H. Y., Chen, Z. H., and Savarese, T. M. Codeletion of the genes for p16INK4. methylthioadenosine phosphorylase, interferon-α*1,* interferon-β1, and other 9p21 markers in human malignant cell lines. Cancer. Genet. Cytogenet., in press, 1996

22. Schlenk, F. Methylthioadenosine. *In:* A. Meister (ed.), Advances in Enzymology and Related Areas in Molecular Biology, pp. 195–265. New York: John Wiley & Sons, 1983.

23. Savarese, T. M., Crabtree, G. W., and Parks, R. E., Jr. Methylthioadenosine phosphorylase-I. Substrate activity of 5'-deoxyadenosine with the enzyme from Sarcoma 180 cells. Biochem. Pharmacol., *30:* 189–199, 1981.

24. Kamatani, N., and Carson, D. A. Dependence of adenine production upon polyamine synthesis in cultured human lymphoblasts. Biochim. Biophys. Acta, *675:* 344–350, 1981.

25. Williams-Ashman, H. G., Seidenfeld, J., and Galletti, P. Trends in the biochemical pharmacology of 5'-deoxy-5'-methylthioadenosine. Biochem. Pharmacol., *31:* 277–288, 1982.

26. Kamatani, N., and Carson, D. A. Abnormal regulation of methylthioadenosine and polyamine metabolism in methylthioadenosine phosphorylase-deficient human leukemia cell lines. Cancer Res., *40:* 4178–4182, 1980.

27. Allegra, C. J., Hoang, K., Yeh, G. C., Drake, J. C., and Baram, J. Evidence for direct inhibition of *de novo* purine synthesis in human MCF-7 breast cells as a principal mode of metabolic inhibition by methotrexate. J. Biol. Chem., *262:* 13520–13526, 1987.

28. Kamatani, N., Nelson-Rees, W. A., and Carson, D. A. Selective killing of human malignant cell lines deficient in methylthioadenosine phosphorylase, a purine metabolic enzyme. Proc. Natl. Acad. Sci. USA, *78:* 1219–1223, 1981.

29. Nobori, T., Karras, J. G., Ragione, F. D., Waltz, T. A., Chen, P. P., and Carson, D. A. Absence of methylthioadenosine phosphorylase in human gliomas. Cancer Res., *51:* 3193–3197, 1991.

30. Nobori, T., Szinai, I., Amox, D., Parker, B., Olopade, O. I., Buchhagen, D. L., and Carson, D. A. Methylthioadenosine phosphorylase deficiency in human non-small cell lung cancers. Cancer Res., *53:* 1098–1101, 1993.

31. Gresser, I. Antitumor effects of interferon. Acta Oncol., *28:* 347–353, 1989.

32. Stiles, G. L., Caron, M. C., and Lefkowitz, R. J. β-adrenergic receptors: biochemical mechanisms of physiological regulation. Physiol. Rev., *64:* 661–743, 1984.

33. Fountain, J. W., Karayiorgou, M., Ernstoff, M. S., Kirkwood, J. M., Vlock, D. R., Titus-Ernstoff, L., Bouchard, B., Vijayasaradhi, S., Houghton, A. N., Lahti, J., Kidd, V. J., Housman, D. E., and Dracopoli, N. C. Homozygous deletions within human chromosome band 9p21 in melanoma. Proc. Natl. Acad. Sci. USA, *98:* 10557–10561, 1992.

34. Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., *72:* 248–254, 1976.

35. Kwiatkowski, D. J., Armour, J., Bale, A. E., Fountain, J. W., Goudie, D., Haines, J. L., Knowles, M. A., Pilz, A., Slaugenhaupt, S., and Povey, S. Report on the second international workshop on human chromosome 9. Cytogenet. Cell Genet., *64:* 94–103, 1993.

36. Murty, V. V. V. S., Bouchard, B., Mathew, S., Vijayasaradhi, S., and Houghton, A. N. Assignment of the human *TYRP* (brown) locus to chromosome region 9p23 by nonradioactive *in situ* hybridization. Genomics, *3:* 227–229, 1992.

37. Allegra, C. J., Drake, J. C., Jolivet, J., and Chabner, B. A. Inhibition of phosphoribosyl-aminoimidazolecarboxamide transformylase by methotrexate and dihydrofolic acid polyglutamates. Proc. Natl. Acad. Sci. USA, *82:* 4881–4885, 1985.

38. Allegra, C. J., Drake, J. C., Jolivet, J., and Chabner, B. A. Inhibition of the folate-dependent enzymes of the *de novo* purine synthesis and folate interconverting enzymes by methotrexate polyglutamates (MTX-PGs). Proc. Am. Assoc. Cancer Res., *25:* 6, 1984.

39. Jackson, R. C., and Grindey, G. B. The biochemical basis for methotrexate cytotoxicity. *In:* F. M. Sirotnak, J. J. Burchall, and W. D. Ensmigner (eds.) Folate Antagonists as Therapeutic Agents, Vol. 1, pp. 289–315. New York: Academic Press, Inc., 1984.

40. Chu, S-H., Ho, L., Chu, E., Savarese, T., Chen, Z. H., and Chu, M. Y. 5'-Halogenated formycins as inhibitors of 5'-deoxy-5'-methylthioadenosine phosphorylase: protection of cells against the growth-inhibitory activity of 5'-halogenated adenosines. Nucleosides & Nucleotides, *5:* 185–200, 1986.

41. Gordon, R. B., Blackwell, K., and Emmerson, B. T. Synthesis of purines in human lymphoblast cells deficient in methylthioadenosine phosphorylase activity. Biochim. Biophys. Acta, *927:* 1–7, 1987.

42. Trackman, P. C., and Abeles, R. H. Methionine synthesis from 5'-S-methylthioadenosine. J. Biol. Chem., *258:* 6717–6720, 1983.

43. Beardsley, G. P., Moroson, B. A., Taylor, E. C., and Moran, R. G. A new folate antimetabolite, 5,10-dideaza-5,6,7,8-tetrahydrofolate, is a potent inhibitor of *de novo* purine synthesis. J. Biol. Chem., *264:* 328–333, 1989.

44. Ray, M. S., Muggia, F. M., Leichman, C. G., Grunberg, S. M., Nelson, R. L., Dyke, R. W., and Moran, R. G. Phase I study of (6R)-5,10-dideazatetrahydrofolate: a folate antimetabolite inhibitory to *de novo* purine synthesis. J. Natl. Cancer Inst., *85:* 1154–1159, 1993.

45. Toohey, J. I. Methylthioadenosine nucleoside phosphorylase deficiency in methyl-thio-dependent cancer cells. Biochem. Biophys. Res. Commun., *83:* 27–35, 1978.

46. Riscoe, M. K., and Ferro, A. J. 5-Methylthioribose: its effects and function in mammalian cells. J. Biol. Chem., *259:* 5465–5471, 1984.

47. Savarese, T. M., Cannistra, A. J., Parks, R. E., Jr., Secrist, J. A. III, Shortnacy, A. T., and Montgomery, J. A. 5'-Deoxy-5'-methylthioadenosine phosphorylase—IV. Biological activity of 2-fluoroadenine-substituted 5'-deoxy-5'-methylthioadenosine analogs. Biochem. Pharmacol., *36:* 1881–1983, 1987.

48. Miyakoshi, J., Dobler, K. D., Allalunis-Turner, J., McKean, J. D. S., Petruk, K., Allen, P. B. R., Aronyk, K. N., Weir, B., Huyser-Wierenga, D., Fulton, D., Urtasun, R. C., and Day, R. S. III. Absence of IFNA and IFNB genes from human malignant glioma cell lines and lack of correlation with cellular sensitivity to interferons. Cancer Res., *50:* 278–283, 1990.

49. Schein, P. S., Lavin, P. T., Moertel, C. G., Frytak, S., Hahn, R. G., O'Connell, M. J., Reitemeier, R. J., Rubin, J., Schutt, A. J., Weiland, L. H., Kalser, M., Barkin, J., Lessner, H., Mann-Kaplan, R., Redhammer, D., Silverman, M., Troner, M., Douglass, H. O., Jr., Milliron, S., Lokich, J., Brooks, J., Chaffe, J., Like, A., Zamcheck, N., Ramming, K., Bateman, J., Spiro, H., Livstone, E., and Knowlton, A. Randomized phase II clinical trial of adriamycin, methotrexate, and actinomycin-D in advanced measurable pancreatic carcinoma: a Gastrointestinal Tumor Study Group report. Cancer (Phila.), *42:* 19–22, 1978.