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Glucuronidation Associated with Intrinsic Resistance to Mycophenolic Acid in Human Colorectal Carcinoma Cells

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ABSTRACT

The *in vivo* efficacy of the antitumor, immunosuppressive antibiotic mycophenolic acid is known to be limited by its rapid conversion to the biologically inactive 7-*O*-glucuronide, catalyzed by UDP-glucuronosyl transferase activity, which is widely distributed among normal tissues, including intestinal epithelium. We have found that mycophenolic acid is also converted to its glucuronide by several lines of human colorectal carcinoma cells, including HT29, Lovo, and Colo-205. In contrast, malignant cell lines not of colorectal origin, including EMT6, HeLa, and SKOV3, showed no ability to metabolize mycophenolic acid. The 7-amino derivative of mycophenolic acid was not metabolized by HT29 cells. This compound was less potent than mycophenolic acid *versus* EMT6 and HeLa cells but showed inhibitory activity against HT29 cells comparable with the parent antibiotic. The rapid metabolism of mycophenolic acid by HT29 cells was associated with a markedly lower sensitivity to both the antiproliferative activity of the drug and to its ability to inhibit GTP synthesis, compared with cells lacking the capacity for significant glucuronidation. After an initial decline in cellular GTP in HT29 cells induced by mycophenolic acid, there was a progressive recovery in GTP over 48 h, accompanying the metabolism of the antibiotic. This recovery process was not observed in EMT6 cells. It is suggested that glucuronosyl transferase activity may occur widely in colorectal cancer cells and could contribute to resistance to drugs that are susceptible to inactivation by glucuronide conjugation.

INTRODUCTION

Drug resistance, intrinsic or acquired, is a common cause of the failure of chemotherapeutic agents to achieve worthwhile responses in human malignant disease. Drug inactivation by metabolism within tumor cells is recognized as an important mechanism of drug resistance (1). The most frequently reported biochemical reaction causing drug inactivation involves the conjugation of electrophilic compounds with glutathione catalyzed by glutathione *S*-transferases (2). Another major route of metabolism of xenobiotics results in the conjugation of nucleophilic groups containing oxygen, nitrogen, sulphur, or carbon to glucuronic acid catalyzed by a broad family of UDPGTs[2] (EC 1.1.205; Ref. 3). These enzymes are widely distributed among normal tissues of the body including liver, lung, skin, kidney, and the epithelial cells of the gastrointestinal tract (3, 4). Despite the importance of glucuronidation as a means of drug inactivation, it appears to have received relatively little attention as a possible mechanism of resistance in malignant cells. However, resistance to daunomycin in a variant of the murine leukemic cell line P338 has been attributed to the inactivation of the cytotoxic metabolite daunorubicinol by elevated UDPGT activity (5). The antitumor and immunosuppressive antibiotic MPA is a specific and direct inhibitor of IMP dehydrogenase (EC 1.2.1.14; Ref. 6), the rate-limiting enzyme in the biosynthesis of guanine nucleotides (7), and of the proliferation of many cell types (8, 9). The inhibition of IMP dehydrogenase results in a marked depression in the cellular concentrations of guanine nucleotides, leading to the arrest of DNA synthesis and the interruption of signal transduction events that involve GTP (6, 10, 11). While examining the responses of several lines of cultured human colorectal cancer cells to MPA, we were struck by the relative resistance of these cells compared with other cell types. MPA is known to be subject to rapid first pass metabolism *in vivo* to the biologically inactive *O*-glucuronide (12). Following recent reports of the ability of the colorectal carcinoma lines Caco-2 and Colo-205 to convert *p*-nitrophenol to the *O*-glucuronide (13, 14), we initiated a study to determine whether colorectal cell lines could inactivate MPA by a similar mechanism. We also examined one mouse and two human malignant cell lines, not of colorectal origin, for evidence of their ability to metabolize MPA.

MATERIALS AND METHODS

Materials. 8-[^{14}C]Hypoxanthine (52 mCi/mmol) was obtained from Amersham International, tri-*N*-octylamine and Freon 11 from Aldrich Chemical Co. (Gillingham, Dorset, United Kingdom), β-glucuronidase (Type H-1) from Sigma Chemical Co., Ltd. (Poole, Dorset, United Kingdom), and trypsin (2.5% in normal saline) from GIBCO-BRL Life Technologies, Ltd. (Paisley, Scotland). MPA and its 7-amino (MPAN) and 7-*O*-glucuronide (MPAG) derivatives were prepared at Zeneca Pharmaceuticals.

Cell Culture. EMT6 (mouse mammary carcinoma), HeLa (human cervical carcinoma), SKOV3 (human ovarian carcinoma), and HT29 (human colorectal carcinoma) cells were grown in Eagle's MEM (GIBCO-BRL) supplemented with 8.6% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 1% glutamine, and 1% nonessential amino acids. Colo-205 cells were grown in RPMI 1640 (GIBCO) supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% glutamine. LoVo cells (human colorectal carcinoma) were grown in DMEM (GIBCO) supplemented as for RPMI 1640. All incubations were carried out in triplicate at 37°C in an atmosphere of air and 5% CO_2, under which conditions the various cell lines appeared to grow optimally. For the determination of the effects of compounds on the increase in cell numbers, EMT6 and HeLa cultures were initiated with 10^4 cells, and HT29 cultures with 10^5 cells, respectively, and incubated for 48 h. The increases in cell numbers were measured over the next 48 h; the medium was decanted from each well, followed by the addition of 150 μl/well trypsin solution for approximately 1 min at 37°C. Growth medium (1 ml) was then added to each well. The resuspended cells were diluted approximately 15-fold with isotonic buffer solution, and the cell numbers were measured with a Coulter counter (Model ZM) at lower and upper thresholds of 10 and 99.9, respectively, and an attenuation of 32. All determinations of cell numbers were carried out on triplicate cultures.

Nucleotide Synthesis. Cells were seeded into flasks (2×10^5 EMT6 cells and 10^6 HT29 cells) and grown overnight. After labeling the cells for 2 h (incubations were done at least three times) with 8-[^{14}C]hypoxanthine (1 μCi/ml of medium), the medium was removed, and the cells were rinsed twice with ice-cold PBS, followed by 10% ice-cold trichloracetic acid. The acid-soluble nucleotide pool was left to extract for 1 h at 0°C. Precipitated protein was removed by centrifugation, and the supernatants were mixed with an equal volume of Freon 11 containing 0.5 M tri-*N*-octylamine to neutralize and extract the trichloracetic acid. Resolution of GTP as a single peak was achieved on a Partisil 10 SAX column, 15 × 0.46 cm, using isocratic elution with 0.6 M ammonium phosphate buffer (pH 3.6) at a flow rate of 1.5–2.0 ml/min. The radioactivity of the effluent stream was monitored with a Berthold LB506-C detector, and the UV absorbance was monitored with a Milton Roy

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[2] The abbreviations used are: UDPGT, UDP-glucuronosyl transferase; MPA, mycophenolic acid; MPAN, 7-amino derivative of MPA; MPAG, 7-*O*-glucuronide derivative of MPA; HPLC, high-performance liquid chromatography.

SpectroMonitor 3100. The retention time for GTP was determined using pure GTP (Sigma Chemical Co.)

Metabolism of MPA. In the initial experiments, HT29 cells were seeded at 10^6 cells per well in 6-well plates and grown for 16 h before the addition of MPA. All incubations were carried out in triplicate and continued for 48 h. After the indicated periods, the medium was removed from each well, and 0.5 ml aliquots were mixed with 1 M HCl and left for 1 h at room temperature. After centrifugation, 145-μl samples of the supernatants were analyzed directly by HPLC using a Spherisorb ODS1 column (5 μm particle size, 10 cm × 4.6 mm). MPA and its *O*-glucuronide were resolved using gradient elution between 30 and 70% methanol in water containing 0.1% trifluoroacetic acid. The retention times for MPA, MPAN, and synthetic MPAG were 13, 12.5, and 9 min, respectively. The peaks containing MPA and the glucuronide were characterized by electrospray mass spectrometry. The kinetic parameters for the metabolism of MPA by HT29, Colo-205, and Lovo cells were determined by seeding 5×10^5 cells into each well in 24-well plates and incubating overnight before the addition of various concentrations of MPA. Medium was removed at intervals over 16 h and analyzed for MPA and MPAG. The values obtained at the 7-h time point, over which period the rates of conversion to MPAG were linear, were used to derive the parameters from Lineweaver-Burk plots fitted by the method of least squares. To determine whether three cell lines not of colorectal origin (EMT6, HeLa, and SKOV3) were capable of converting MPA to its glucuronide, the cells were grown to confluency and then incubated with 31 μM MPA for 48 h before removing the medium for HPLC analysis.

Treatment of the Biosynthetic *O*-Glucuronide of MPA with β-Glucuronidase. MPA was incubated with preconfluent cultures of HT29 cells for 48 h. The media from one set of cultures were analyzed for MPA and its metabolite as described above, whereas the media from a replicate set were incubated for 24 h with an equal volume of 0.1 M sodium acetate buffer (pH 5.0) containing 1 mg/ml of β-glucuronidase. After acidification and centrifugation, the supernatants were analyzed by HPLC as described above.

RESULTS

Effects of MPA and Derivatives on the Proliferation of Human Colorectal Adenocarcinoma Cells (HT29), Murine Mammary Carcinoma Cells (EMT6), and HeLa Cells. Table 1 indicates that the proliferation of HT29 cells was substantially more resistant to MPA than that of either EMT6 or HeLa cells; the IC_{50} for the colorectal cells was 54- and 16-fold higher than the values for EMT6 and HeLa cells, respectively. The IC_{50}s for MPAN against EMT6 cells and HeLa cells were 6.7- and 7.9-fold higher than the values for MPA, respectively, whereas the compound exhibited comparable potency with MPA against HT29 cells. As expected, MPAG had no effect on the proliferation of EMT6 or HT29 cells.

Inhibition of GTP Synthesis in HT29 and EMT6 Cells by MPA and MPAN. The biochemical activity of MPA in cultured cells can be conveniently assessed by measuring the inhibition of the incorporation of radiolabeled hypoxanthine into the cytoplasmic pool of GTP. Cultures of EMT6 and HT29 cells were labeled for 2 h with [^{14}C]hypoxanthine, with or without MPA, and the nucleotide content of the acid extract of the cell layers was analyzed by HPLC as described. The data in Table 1 indicate that the potency of MPA against GTP synthesis in EMT6 cells was 10 times higher than that against HT29 cells. In contrast, the IC_{50}s for MPAN against GTP synthesis in EMT6 and HT29 cells differed only by 2.2-fold.

Table 1 *Activities of MPA, and MPAN, and MPAG against EMT6, HT29 and HeLa cells*[a]

Assay	IC_{50} μM		
	MPA	MPAN	MPAG
EMT6			
Proliferation	0.24	1.60	>100
GTP synthesis	0.075	2.5	ND
HT29			
Proliferation	12.9	7.3	>100
GTP synthesis	0.75	5.6	ND
HeLa			
Proliferation	0.8	6.3	ND

[a] The cultures were established, and the effects of the compounds on the increases in cell numbers over 48 h were determined as described in "Materials and Methods." The effects on the biosynthesis of GTP were determined by measuring the incorporation of 8-[^{14}C]hypoxanthine into GTP over 2 h as described in "Materials and Methods." ND, not determined.

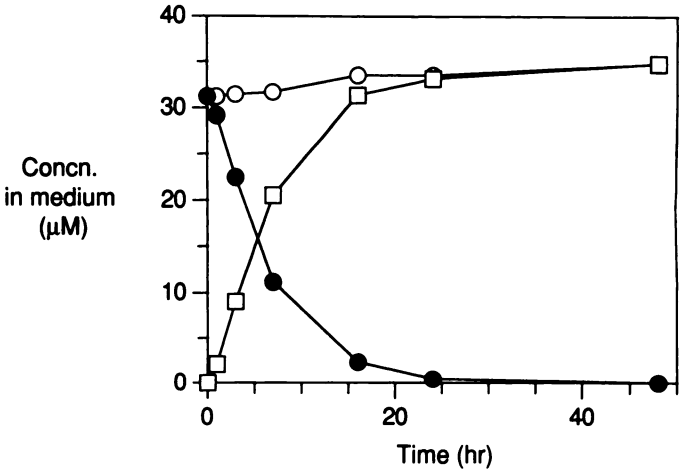

Fig. 1. Metabolism of MPA by monolayer cultures of HT29 cells. The cells were grown to preconfluency before the addition of 31.25 μM MPA. After the indicated intervals, the medium was harvested and assayed for its content of MPA and the corresponding 7-*O*-glucuronide derivative (MPAG). ●, MPA in medium incubated with monolayer of cells; ○, MPA in medium incubated without cells; □, MPAG released into medium incubated with monolayer of cells. *Concn.*, concentration.

Table 2 *Determination of apparent K_m and V_{max} for the conversion of mycophenolic acid to the 7-O-glucuronide by human colorectal cacinoma cell lines*[a]

Cell line	K_m (μM)	V_{max} (nmole of MPAG synthesized/h)
HT29	45	5.8
Colo-205	77	14.3
Lovo	400	2.9

[a] Approximately 10^6 cells were incubated in culture medium with various concentrations of MPA. All incubations were carried out in triplicate. The medium was removed at intervals up to 16 h and analyzed for MPA and MPAG as described in "Materials and Methods." The kinetic parameters were obtained from Lineweaver-Burk plots of the data from the 7-h time point fitted by method of least squares.

Metabolism of MPA to Its *O*-Glucuronide by Human Colorectal Carcinoma Cell Lines. MPA is rapidly converted *in vivo* to the biologically inactive glucuronide conjugate at the phenolic hydroxyl group of position 7 (12). The reported ability of a human colorectal adenocarcinoma cell lines to convert *p*-nitrophenol to *p*-nitrophenylglucuronide (13, 14) suggested that the relative resistance of HT29 cells to the inhibitory effects of MPA might be associated with inactivation of the compound by glucuronidation. Preconfluent monolayer cultures of HT29 cells were incubated over 48 h in growth medium initially containing 31 μM MPA, and the medium was removed at intervals for HPLC analysis. Fig. 1 shows that MPA was rapidly depleted from the medium, and that this was accompanied by the appearance of a compound with the HPLC retention characteristics of the chemically synthesized 7-*O*-glucuronide of MPA. The formation of the glucuronide was confirmed in two ways: (*a*) growth medium, initially containing MPA, was incubated with a monolayer of HT29 cells for 48 h. The medium was decanted off, a sample retained from HPLC analysis, and the remainder was incubated with or without β-glucuronidase for 24 h and chromatographed. The peak corresponding to the glucuronide of MPA disappeared from medium treated with β-glucuronidase and was replaced quantitatively by a

Table 3 *Changes in cellular GTP levels in HT29 and EMT6 cells incubated with MPA*[a]

	GTP (nmol/10^5 cells) at					
	2 h		24 h		48 h	
MPA (μM)	EMT6	HT29	EMT6	HT29	EMT6	HT29
0	2.2	2.1	1.7	1.8	2.8	2.2
1.55	NM	ND	NM	ND	NM	ND
3.1	ND	0.7	ND	1.8	ND	2.5
31.0	ND	0.6	ND	0.4	ND	2.3

[a] Overnight cultures of EMT6 and HT29 cells were incubated with MPA for the indicated periods before the monolayers were extracted with ice-cold 10% trichloracetic acid, and the GTP contents of the neutralized extracts were determined as described in "Materials and Methods." NM, GTP contents of the extracts were below detectable levels. ND, GTP contents not determined.

peak corresponding to MPA, whereas there was no change in the glucuronide peak in the untreated medium; and (*b*) medium containing MPA that had been incubated with HT29 cells for 48 h was deproteinized and subjected to HPLC, followed by electrospray mass spectrometry. No peak with a mass corresponding to MPA was detected, whereas a peak containing an ion with a mass of 519, corresponding to the *O*-glucuronide of MPA plus one sodium atom, was evident. When HT29 cells were incubated in growth medium containing MPAN, no depletion of this compound from the medium was detected over 48 h, indicating that the 7-amino substitution prevented the formation of a glucuronide derivative.

We examined the dependence of the rate of synthesis of the glucuronide of MPA on the initial concentration of MPA in the culture medium by HT29 cells and two other human colorectal carcinoma cell lines, Lovo and Colo-205, over 16 h and plotted a Lineweaver-Burk lines to the experimental points from the 7-h time point by the method of least squares. The apparent K_m and V_{max} values (Table 2) indicate that HT29 cells were the most efficient of the three lines in metabolizing MPA, followed by Colo 205 cells. Lovo cells also carried out the conversion, but the apparent kinetic parameters were significantly inferior to those for the other two lines. In contrast, three cell lines not of colorectal origin [EMT6 (mouse mammary carcinoma), HeLa (human cervical carcinoma), and SKOV3 (human ovarian carcinoma) cells] showed no measurable ability to metabolize MPA over 48 h; the concentration of MPA in the medium remained unchanged, and MPAG could not be detected in any of the cultures (not shown).

Recovery of GTP Levels in HT29 Cells on Prolonged Incubation with MPA. The inactivation of MPA by HT29 cells suggested that this might permit the recovery of cellular GTP after the initial phase of depletion. Overnight cultures of HT29 cells (seeding density, 5×10^5 cells/ml of medium) and EMT6 cells (5×10^4 cells/ml) were incubated for up to 48 h with various concentrations of MPA, and the cells were harvested at intervals for nucleotide analysis. Table 3 indicates that after the initial depletion of cellular GTP, the level of GTP in HT29 cells recovered progressively over the next 46 h, even at the highest concentration of MPA tested (31 μM). In contrast, in EMT6 cells treated with 1.55 μM MPA, the GTP content remained below detectable levels for 46 h after the initial depletion.

DISCUSSION

Glucuronidation is a major route for the metabolic inactivation of a wide range of drugs and some endogenous substances. Unlike another common pathway of xenobiotic metabolism (the conjugation of electrophilic compounds with glutathione), glucuronidation has received little consideration as a possible contributor to drug resistance in malignant cells. The finding that Caco-2 and Colo-205 cells, both of which derive from human colorectal carcinomas, glucuronidate *p*-nitrophenol (13, 14) suggested that the greater resistance to MPA of HT29 cells (which are also of human colorectal carcinoma provenance) compared with EMT6 cells (mouse mammary carcinoma) and HeLa cells (human cervical carcinoma) might be due to the inactivation of the antibiotic by a similar mechanism. The IC_{50} of MPA *versus* the proliferation of HT29 cells over 48 h was approximately 54-fold greater than that *versus* EMT6 cells, and 16-fold greater than the value for HeLa cells. IC_{50}s reported for MPA against the proliferation of other human cell lines not of colorectal origin are comparable with those for EMT6 cells and HeLa cells [*e.g.*, HO melanoma cells, ~0.5 μM (15); CaPan-2, 0.8 μM (8); CaLu-3, 0.35 μM (8); FIB, 0.32 μM (9)]. The relative resistance of HT29 cells to MPA was also evident in the 10-fold higher IC_{50} for the inhibition of GTP biosynthesis over a 2-h period compared with the corresponding value for EMT6 cells.

The evidence in this report suggests that the relative resistance of HT29 cells to MPA in comparison with noncolorectal lines is probably associated with their ability to convert MPA to the biologically inactive MPAG because the more sensitive EMT6 and HeLa lines showed no evidence of this conversion. The human ovarian carcinoma line SKOV3 also did not convert MPA to the glucuronide, although the sensitivity of this line to MPA remains to be assessed. The rapid glucuronidation of MPA *in vivo* by nontumor tissues has previously been assumed to be the main factor in limiting its clinical efficacy in immunosuppressive therapy (9) and against tumor growth (16). Considerable effort has therefore been expended to find derivatives of MPA with greater metabolic stability. The 7-amino derivative of MPA, which is not subject to metabolic inactivation either *in vitro* or *in vivo*,[3] was less effective than MPA *versus* EMT6 and HeLa cells. However, the potency of MPAN *versus* HT29 cells was comparable with that of MPA, presumably because of its resistance to metabolism by the colorectal cells. The progressive recovery of cellular GTP levels in HT29 cells, which followed the initial decline induced by MPA, is also likely to be linked to the rapid glucuronidation of MPA because there was no similar recovery of GTP in EMT6 cells treated with MPA. Two other human colorectal carcinoma cell lines (Lovo and Colo-205) also converted MPA to its glucuronide. Currently, eight isoforms of human UDPGTs have been identified that have a range of substrate specificities (3). As yet, there is no information as to the spectrum of UDPGTs that are present in colorectal cancer cells, but it can be anticipated that the *O*-glucuronidation of MPA would be catalyzed by one or more of the enzymes that accept phenolic substrates, such as UGT1*6 and UGT1*02 (3). Information is also lacking on the levels of UDPGT activities in various colorectal cancer cell lines, although our data on the relative abilities of HT29, Colo-205, and Lovo cell lines to metabolize MPA suggests that there may be significant differences in UDGPT activities among colorectal tumors.

The present report, together with those demonstrating UDPGT activity in Caco-2 and Colo-205 cells (13, 14), suggests that the capacity to inactivate appropriate substrates by glucuronidation may not be uncommon among colorectal cancers, whereas it may only occur

[3] T. J. Franklin, unpublished data.

sporadically in tumors derived from other tissues. The diversity of UDPGTs and their wide-ranging substrate specificities suggest that consideration should be given to the likely susceptibility to glucuronidation in the design of novel antitumor agents directed against colorectal cancer.

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