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CARBOXYPEPTIDASE G2 AND TRIMETREXATE CAUSE GROWTH DELAY OF HUMAN COLONIC CANCER CELLS *IN VITRO*

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Abstract—MAWI colonic cancer cells respond to sequential treatment *in vitro* with carboxypeptidase G2 and trimetrexate by a delay in cell growth as measured by cell numbers, but an increase in incorporation of 75-Se-selenomethionine per cell. The cells are not methionine auxotrophs.

In 1971, Bertino *et al.* [1] reported successful inhibition of growth of L1210, L5178Y and Walker 256 tumour cells *in vitro* resulting from their exposure to 0.25 units/mL of carboxypeptidase G1, an enzyme which inactivates reduced and non-reduced folates by deglutamylation. The enzyme carboxypeptidase G2 [2] shares the same specificity, and since it retains its activity linked to an antibody [3] can be targeted to tumour cells *in vivo*. 5-Methyl-tetrahydrofolate, which constitutes a high proportion of the folates present in serum, would be the prime subject of attack by extracellular tumour-located carboxypeptidase: 5-methyl-tetrahydrofolate deprivation should lower the tetrahydrofolate available to the cell via methionine synthetase. The MAWI cell line, derived from a moderately differentiated human colonic adenocarcinoma which has been passaged repeatedly in nu/nu mice [4] provides a model to study growth delay in the presence of carboxypeptidase G2 and trimetrexate, a folate antagonist lacking a glutamate moiety and thus unimpaired by the enzyme. The synthesis of DNA in Friend leukaemia cells becomes compromised at folate levels of 0.3 ng (0.6 pmol)/10^6 cells, i.e. around one-tenth those for efficient growth [5]. The deprivation needs to be sustained for a cytotoxic effect. Mouse hepatoma cells deprived of supplements for 5 days so that their methylene tetrahydrofolate levels fall to one-tenth the concentration of folate replete cells (i.e. to 0.5 pmol/10^6 cells) revert to normal growth after only 4 hr of leucovorin rescue [6]. Colonic cancer cells are likely to be resilient to direct folate deprivation since their inherent folate levels can be higher than in normal colon or liver tissues [7] and elevated levels of thymidine kinase [8] may accentuate dependence on salvage pathways for thymidine. However, if distortion of cellular metabolism in colonic cancer cells by carboxypeptidase G2 can be demonstrated, the selective deprivation engendered by antibody-targeted enzyme *in vivo* can be used as a starting point to design regimens to treat this recalcitrant disease by combination of the enzyme with antipyrimidine or antipurine antagonists.

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MATERIALS AND METHODS

Tritiated thymidine and 75-Se-selenomethionine were obtained from Amersham International (Amersham, U.K.). Culture flasks and plates were supplied by Costar. Media for cell culture and foetal calf serum were supplied by Gibco (Uxbridge, U.K.). Cells were maintained routinely in medium containing 10% heat inactivated foetal calf serum, 2 mM L-glutamine, 20 mM HEPES, 100 units of penicillin and 100 μg of streptomycin per mL. Trimetrexate glucuronate (TMQ) was a gift from Warner Lambert Ltd. It was administered to cells in aqueous solution.

Primary culture of MAWI tumour cells. A human colonic tumour was grown as a xenograft (MAWI) in outbred nude (nu/nu) mice [4]. Tumour tissue was cut up into fragments and the MAWI cell line was developed from a spill culture, i.e. from cells released from washing the tissue fragments. Cells were plated out in 25 cm^2 flasks at 10^6 cells/flask in primary culture medium (DMEM + 20% foetal calf serum + 200 units of penicillin + 200 μg streptomycin/mL). Twenty-four hours later flasks were exposed to fresh primary culture medium containing heat-inactivated rabbit anti-nude mouse serum (1/30 dilution in medium). Established cell lines, seeded at 10^5 cells per 25 cm^2 flask reached confluency at 7–10 days.

Production of rabbit anti-nude mouse spleen antibodies. Freshly excised nude mouse spleen cells, teased apart, were injected i.v. (10^8 cells) into rabbits (three injections at 2-week intervals). Rabbit serum obtained 2 weeks after the final injection was heated to inactivate complement, (56°, 30 min).

Treatment of MAWI cells in RPMI 1640 *with carboxypeptidase G2 and Trimetrexate glucuronate (TMQ).* (i) MAWI cells received carboxypeptidase G2 treatment for 96 hr, TMQ treatment for the final 48 hr and 75-Se-selenomethionine for 19 hr. On day

1, MAWI cells (10^5) were plated out from maintenance medium into RPMI 1640 + 10% foetal calf serum in 1-mL wells. On days 2 and 3, the cells, apart from controls, were treated in triplicate with 3 units/mL or 5 units/mL of carboxypeptidase G2. On day 4, groups of cells were treated with 0, 50, 75 or 100 μM TMQ (final concentration per well). Five hours after the TMQ all cells received 75-Se-selenomethionine (0.5 μCi/well). On day 5, cells were washed three times with saline, trypsinized and counted in a LKB-Wallac 8000 γ-counter. The 75-Se-selenomethionine incorporated was expressed as the per cent of the counts per min incorporated by control cells. Concentrations of stock solutions in media were adjusted so that constant volumes of 100 μL were added to wells.

(ii) On day 1, MAWI cells were set up (5×10^4) in RPMI 1640 (2 mL). The test wells [9] were treated for days 2–5 with 10 units of carboxypeptidase G2 in the above medium, (2 mL) while control cells were kept in medium alone. On day 5, the test wells were treated with TMQ (50 μM) also. 75-Se-selenomethionine (1 μCi) was added 5 hr later to three test and three control wells. On day 6, cells from these wells were washed, harvested and counted as before. The remaining test and control wells were replenished daily with RPMI 1640 to determine the capacity of the test wells to regrow after the removal of the enzyme and TMQ. On days 7, 10 and 11 cells which had received isotope as above on days 6, 9 and 10, respectively, were harvested and counted.

Response of MAWI cells to methionine or homocysteine in EMEM. MAWI cells (5×10^4) were plated out in EMEM medium (2 mL) or EMEM supplemented with L-homocysteine or with L-methionine (15 mg/L). The relative cell numbers were compared by haemocytometry after 5 days' growth. The comparative incorporation of tritiated thymidine (sp. act. 5 Ci/mmol, 1 μCi/well, 3 hr pulse) for cells in EMEM or EMEM and homocysteine was measured and compared to the incorporation of the isotope by cells growing in DMEM under standard conditions. The cells were washed three times with saline, trypsinized and centrifuged. The pellet was digested in 2% sodium dodecyl sulphate overnight, scintillation fluid (Optiphase Hisafe, LBK) was added and disintegrations determined with a Beckmann scintillation counter (LS 7500).

Statistical analyses of cellular responses to enzyme and TMQ. (i) A deviance table was constructed to show all terms which might be included in a saturated log-linear model of per cent survival rates [10], and expected survival rates calculated from a selected model were compared with the experimental data.

(ii) An analysis of variance table was produced for the cell numbers/10^6 cells for test and control groups, the sources of variation being considered as time, treatment and time × treatment.

RESULTS

Establishment of MAWI cell line

MAWI xenografts, maintained over many passages resemble the histology of the original human colon adenocarcinoma, with well differentiated goblet cells, low columnar cells and signet ring cells. Expression of carcinoembryonic antigen has been retained.

The cell line was characterized as a "low-producer" of carcinoembryonic antigen: early cultures released 60–70 ng/mL/10^6 cells, but this diminished beyond the fourth passage to 20 ng/mL/10^6 cells remaining steady thereafter [11]. Tissue typing identified antigens consistent with the expression of HLA A, B and C [11]. Injection of 5×10^7 MAWI cells into the flanks of nu/nu mice resulted in poorly differentiated solid xenografts in contrast to the original histology. The *in vitro* doubling time of MAWI cells varies slightly according to conditions but the shortest doubling time recorded is 18 hr in DMEM primary culture medium.

Cytotoxicity of rabbit anti-nude mouse serum antibodies

In the presence of rabbit complement the antiserum was toxic to nude mouse spleen cells and fibroblasts, while the MAWI tumour cells survived treatment.

Treatment of MAWI cells in RPMI 1640 with carboxypeptidase G2 and TMQ

(i) The greatest inhibition of growth was observed with 100 μM TMQ and 5 units of enzyme per mL, but significant inhibition was apparent in the presence of enzyme alone at 3 units/mL or TMQ alone (50 μM). The agents appeared to act in concert but not additively. Full results are given in Table 1.

Table 1. Treatment of MAWI cells in RPMI 1640 with carboxypeptidase G2 and TMQ: 75-Se-selenomethionine incorporated (cpm as % of untreated control)

Dose of TMQ (μM)	Dose of carboxypeptidase G2 (%) (units/mL) 0	3	5
0	100	57	32
50	70	44	28
75	64	37	26
100	60	33	24

(ii) The cell numbers from haemocytometry and 75-Se-selenomethionine incorporation in the recovery phase after removal of enzyme and TMQ are given in Table 2. Whereas the control cells grew steadily towards confluence and in so doing had incorporated proportionately less isotope per 10^6 cells, the test cell numbers had not increased significantly in 4 days. These cells appeared to be larger and to have increased uptake of isotope per 10^6 cells.

Response of MAWI cells to methionine or homocysteine in EMEM

After 5 days the mean cell count was seven-fold greater in medium containing methionine than in unsupplemented EMEM, but only three-fold greater in medium containing homocysteine. Thymidine

Table 2. Recovery of MAWI cells in RPMI 1640 after treatment with carboxypeptidase G2 (5 units/mL) and TMQ (50 μM)

Time post TMQ		Cell number $\times 10^5$	75-Se cpm per 10^6 cells
24 hr	Test	7.03 ± 0.2	25119 ± 944
	Control	13.76 ± 0.4	26280 ± 831
48 hr	Test	7.6 ± 1.2	29199 ± 3651
	Control	38.6 ± 3.0	13594 ± 1280
72 hr	Test	4.8 ± 1.6	40807 ± 16079
	Control	35.1 ± 8.0	16596 ± 4226
96 hr	Test	4.4 ± 1.0	36552 ± 8948
	Control	38.0 ± 1.4	11022 ± 615

Results are mean ± SD.

Table 3. Response of MAWI cells to methionine or homocysteine in EMEM

	EMEM	EMEM + homocysteine	EMEM + methionine
Mean cell count	1.9×10^7	5.77×10^7	1.30×10^8
Relative count	1	3	7
			DMEM
Mean ^{3}H incorporation (tritiated thymidine)			
(10 determinations)	23548	37491	26466
$\sigma/\sqrt{N}$	5976	7549	3695

incorporation, expressed as the dpm/10^5 cells did not differ notably between cells grown in DMEM and EMEM but was increased somewhat in the EMEM containing homocysteine (Table 3).

Results of statistical analyses on data from Tables 1 *and* 2

The deviance table (Table 4) shows all terms that might be included in a saturated model. The subset of terms selected as the simplest adequate model of the observed survival percentages are marked with an asterisk. This subset includes a minimal model which serves to fix marginal totals determined by the fact that

$$\% \text{ surviving cells} + \% \text{ dead cells} = 100\%$$

Table 4. Deviance table

Term	Degrees of freedom	Deviance	Approximate P-value
(Minimal model)	(6)*	(2)	—
TMQ × CPG2	6	7	0.3
% Survival × TMQ	3*	41	≪0.0005
% Survival × CPG2	2*	182	≪0.0005
% Survival × TMQ × CPG2	6*	42	≪0.0005

* Selected terms

for all treatment combinations.

It also includes explanatory terms which must have associated P-values below the chosen significance (0.05). The expected survival percentages derived from this selected model are given in Table 5. For comparison with the observed data in Table 1. An analysis of variance table (Table 6) is given for data from Table 2.

DISCUSSION

The aim of the experiments undertaken was to establish whether a regimen of carboxypeptidase G2-determined folate deprivation in MAWI human colonic cancer cells, followed by trimetrexate administration would cause a measurable disturbance in cellular metabolism over 4–6 days. The duration of the experiments was chosen to correspond with enzyme retention by antibody targeting

Table 5. Expected per cent survival of MAWI cells in response to carboxypeptidase G2 and TMQ according to selected model

Dose of TMQ (μM)	Dose of carboxypeptidase G2 (units/mL) 0	3 (%)	5
0	97	56	36
50	73	42	27
75	65	38	24
100	60	35	22

Table 6. Analysis of variance of numbers of cells (from Table 2)

Source of variation	df(mv)	ss	ms	vr	Approximate P-value
Time	3	57945	19315	12.25	<0.001
Treatment	1	387795	387795	245.85	≪0.001
Time × treatment	3	70775	23592	14.96	<0.001
Residual	15(1)	23660	1577		
Total	22(1)				

Where df (mv) is degrees of freedom (missing value); ss is sums of squares; ms is mean square; vr is variance ratio.

in vivo. 75-Se-selenomethionine labelling has been shown to correlate well with cell numbers *in vitro* [12]. The accumulation of 75-Se-selenomethionine into MAWI cells was diminished markedly by exposure of the cells for 72 hr to 3–5 units of carboxypeptidase G2 per mL of RPMI 1640 medium. Measurable depression of counts could be achieved by 24 hr exposure of the cells to at least 50 μM TMQ. Despite extensive investigations varying the time, enzyme units and TMQ levels between 2–5 days, 3–5 units and 50–100 μM, respectively, combining the reagents sequentially depressed the cell-labelling at best to a plateau of around 25% control incorporation (representative experiment, Table 1). Similar results, not shown here, were obtained in DMEM. A clearer picture emerged when the growth of the cells was followed after the enzyme and TMQ were removed and the cells were re-exposed to replete medium. While the control cells grew to confluence and apparently required less exogenous methionine, the residual treated cells remained sluggish though their methionine uptake per cell increased, reflected in the isotope levels.

From the deviance table, it can be seen that the simplest model that adequately explains the data must include all terms except TMQ × carboxypeptidase G2 concentrations. If graphs are drawn for the per cent survival rates (y-axis, linear) versus the TMQ μM concentrations (x-axis, log scale) for observed and expected data at concentrations of enzyme, 0, 3 or 5 units/mL (not shown) the "goodness of fit" of our selected model, which is high since P-values associated with terms included are extremely small, is further illustrated by the virtual superimposition of the graphs. The negative slope of the graphs illustrates the dependence of per cent survival on TMQ concentration. This has $P \ll 0.0005$. The dependence of per cent survival on concentration of carboxypeptidase G2 is illustrated by the "stacking" of the graphs, i.e. per cent survival at dose 3 units/mL is completely below dose 0 and per cent survival at dose 5 units/mL is completely below 3 units/mL. This dependence has $P \ll 0.0005$ also. Finally the convergence of the three graphs at higher concentrations of TMQ suggests that the interaction ($P \ll 0.0005$) per cent survival × TMQ × carboxypeptidase G2 is a mutual inhibitory effect. In considering a mutually inhibitory effect it should be borne in mind that at 5 units/mL of carboxypeptidase G2 the graph shows almost zero gradient, or in other words, at high doses of enzyme, increasing the dose of TMQ does little to increase cytotoxicity.

The analysis of variance table (Table 6) shows that the P-value for the main effect (of treatment) is well below the significance level of 0.05.

There is an implicit proviso in our experiments that if low levels of folate cofactors limit the full use of homocysteine, via methyl tetrahydrofolate-homocysteine methyl transferase [13], incorporation of labelled methionine would overestimate cell numbers in relation to controls. Any inherent tendency to methionine dependence would be present in both tests and controls. The comparative rates of growth in DMEM and EMEM supplemented with homocysteine or methionine do not suggest that the cells are total methionine auxotrophs.

The following conclusions can be drawn. The MAWI cell line is relatively resistant to TMQ by comparison with the response of other cell lines [14]. It seems unlikely that TMQ is excluded by the MAWI cells: it is a lipophilic drug which does not depend on methotrexate transport pathways for cell entry [15]. Since naive MAWI cells respond to 2 μM methotrexate treatment (data not shown) the insensitivity to TMQ is surprising. A supposition could be that the dihydrofolate reductase binding in these cells is particularly dependent on polyglutamylated cofactor. A significant disturbance in protein synthesis can be achieved in MAWI cells within 4–6 days' exposure to levels of enzyme which could be sustained at the site of a tumour xenograft *in vivo* by an appropriate antibody [9]. Irreversible cytotoxicity has not been achieved here using the enzyme as a single agent, which was disappointing in view of earlier results [1]. It may be relevant that some cells which had been shown to be particularly responsive to folate deprivation by carboxypeptidase G1 are also methionine dependent, for example L1210 and L5178Y [16]. The Walker 256 rat adenocarcinoma which responds to carboxypeptidase G1 treatment *in vivo* is thymidine kinase deficient, which defect would lower its capacity to rely on thymidine salvage in low folate conditions. The MAWI cells do not manifest these increased susceptibilities.

For malignant cells with fairly low resilience to folate deprivation, antitumour antibody-targeted carboxypeptidase G2 and TMQ given sequentially could be promising partners for selective therapy. More resistant cells, such as those of colonic cancer, may need concomitant treatment with conventional drugs. The increased levels of phosphoribosyl pyrophosphate resulting from folate deprivation [17] should enhance the conversion of 6-mercaptopurine by hypoxanthine-guanine-phosphoribosyl-

transferase [18] making this drug an appropriate partner for the enzyme. An exciting new development has emerged recently whereby the carboxypeptidase-catalysed deglutamylation is used to convert a prodrug into an active alkylating drug. This approach has proved effective in delaying the growth of human choriocarcinoma xenografts in nude mice [19]. Successful transposition to colonic cancer xenografts will be aided by understanding the role of folate deprivation in conjunction with the attack of the released alkylating agent, particularly if a prolonged interval is needed to effect plasma clearance of untargeted enzyme.

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REFERENCES

1. Bertino JR, O'Brien P and McCullough JL, Carboxypeptidase G1: a new approach to antifolate chemotherapy. *Science* **172**: 161–162 1971.
2. Sherwood RF, Melton RG, Alwan SM and Hughes P, Purification and properties of carboxypeptidase G2 from *Pseudomonas* sp. strain RS-16 use of a novel triazine dye affinity method. *Eur J Biochem* **148**: 447–453, 1985.
3. Searle F, Bier C, Buckley RG, Newman S, Pedley RB, Bagshawe KD, Melton RG, Alwan SM and Sherwood RF, The potential of carboxypeptidase G2-antibody conjugates as antitumour agents. I. Preparation of anti-human chorionic gonadotrophin-carboxypeptidase G2 and cytotoxicity of the conjugate against JAR choriocarcinoma cells *in vitro*. *Br J Cancer* **53**: 377–384, 1986.
4. Lewis JCM, Smith PA, Keep PA and Boxer GM, A comparison of the content and immunohistochemical patterns of CEA-like activity in human colorectal tumours and nude mouse xenografts. *Exp Pathol* **24**: 227–235, 1983.
5. Steinberg S, Fonda S, Campbell CL and Hillman RS, The intracellular folate pool: studies of kinetics and functional significance. In: *Chemistry and Biology of Pteridines* (Ed. Blair JA), pp. 1013–1017. Walter de Gruyter and Co., Berlin, 1983.
6. Priest DG, Doig MT and Mangum M, Adaptation of folyl polyglutamates to folate deprivation in mouse hepatoma cells. In: *Chemistry and Biology of Pteridines* (Ed. Blair JA), pp. 965–969. Walter de Gruyter and Co., Berlin, 1983.
7. Baker H, Frank D, Chen T, Feingold S, De Angelis B and Baker ER, Elevated vitamin levels in colonic adenocarcinoma as compared with metastatic liver adenocarcinoma from colon primary and normal adjacent tissue. *Cancer* **47**: 2883–2886, 1981.
8. Weber G, Enzyme programmes in slow and fast colon cancer xenografts. *Cancer Res* **41**: 854–859, 1981.
9. Melton RG, Searle F, Bier C, Boden JA, Pedley RB, Green AJ, Bagshawe KD and Sherwood RF, Antibody-carboxypeptidase conjugates as potential imaging agents. In: *Radiolabelled Antibodies for Imaging and Therapy* (Ed. Srivastava SC), NATO ASI Series Life Sci, Vol. 152, pp. 377–383. Plenum, New York, 1988.
10. McCullagh P and Nelder JA, Generalised linear models. Chapman and Hall, London, 1983.
11. Partridge CS, The kinetics of antibody binding to colorectal cancer cells at 4° and 37°C. PhD thesis. London University, 1986.
12. Brooks CG, Rees RC and Robbins RA, Studies on the microcytotoxicity test. II. The uptake of amino acids ^{3}H-leucine and ^{75}Se-selenomethionine but not nucleosides (^{3}H-thymidine or ^{125}I-iododeoxyuridine) or $^{51}CrO_4^{2-}$ provides a direct and quantitative measure of target cell survival in the presence of lymphoid cells. *J Immunol Methods* **21**: 111–124, 1978.
13. Tautt JW, Anuzewska EL and Koziorowska J, Methionine regulation of *N*-5-methyl tetrahydrofolate homocysteine methyl transferase and its influence on the growth and protein synthesis in normal, neoplastic and transformed cells in culture. *J Natl Cancer Inst* **69**: 9–14, 1982.
14. Diddens H, Niethammer D and Jackson RC, Patterns of cross-resistance to the antifolate drugs trimetrexate, metoprine homofolate and CB 3717 in human lymphoma and osteosarcoma cells resistant to methotrexate. *Cancer Res* **43**: 5286–5292, 1983.
15. Rodenhuis S, McGuire JJ, Narayanan R and Bertino JR, Development of an assay system for the detection and classification of methotrexate resistance in fresh human leukemic cells. *Cancer Res* **46**: 6513–6519, 1986.
16. Hoffman RM, Methionine dependence in cancer cells—a review. *In Vitro* **18**: 421–426, 1982.
17. Ghitis J, Schreiber C and Waxman S, Phosphate-induced phosphoribosyl pyrophosphate elevations to assess deranged folate and purine nucleotide metabolism. *Proc Soc Exp Biol Med* **186**: 90–95, 1987.
18. Bökkerink JP, de Abren RA, Bakker MAH, Hulscher TN, van Baal JM and de Vaan GAM, Dose related effects of methotrexate on purine and pyrimidine nucleotides and on cell-kinetic parameters in MOLT-4 malignant human T lymphocytes. *Biochem Pharmacol* **35**: 3557–3564, 1984.
19. Bagshawe KD, Springer CJ, Searle F, Antoniw P, Sharma SK, Melton RG and Sherwood RF, A cytotoxic agent can be generated selectively at cancer sites. *Br J Cancer* **58**: 700–703, 1988.