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ORIGINAL ARTICLE

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Inhibition of γ-glutamyl transpeptidase activity decreases intracellular cysteine levels in cervical carcinoma

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Abstract *Purpose*: To determine whether gamma-glutamyl transpeptidase (γ-GT) is involved in the maintenance of elevated cysteine levels in cervical carcinoma. *Methods*: Four cervical carcinoma cell lines were tested in vitro for cysteine accumulation and γ-GT levels. The highest and lowest γ-GT-expressing cell lines were used in in vivo experiments to determine the effect of γ-GT inhibition on cysteine levels. *Results*: Treatment of a series of cervical carcinoma cell lines with acivicin decreased intracellular cysteine concentrations. Cysteine depletion was evident in Me180 cells which had the greatest levels of γ-GT activity, and had a more pronounced cysteine decrease in medium with glutathione and cysteine concentrations simulating the in vivo situation. Also investigated were the effects of inhibition of γ-GT activity on intracellular cysteine levels in xenografts grown in severe combined immunodeficient (SCID) mice. With the use of 35 mg/kg of acivicin, γ-GT activity decreased to basal levels of detection in both tumour types and significant decreases in cysteine levels were seen in the high γ-GT-expressing tumours (Me180). Thus, inhibition of γ-GT activity may have therapeutic potential in high-expressing cancers. *Conclusions*: In tumours and cell lines with elevated levels of γ-GT activity, inhibition of this enzyme led to decreases of cysteine levels.

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Introduction

The non-protein sulphydryls (NPSH) glutathione (GSH) and cysteine are able to effect chemical repair of radiation-induced DNA radical sites, particularly under low oxygen conditions that exist in some solid tumours [1, 2]. Increased tumour GSH or cysteine content is therefore a potentially important cause of radiotherapy failure in the clinic [2, 3]. The radioprotective effect of cysteine is estimated to be up to tenfold greater than that of GSH [1], probably because cysteine has improved access to DNA radical sites due to its smaller size and net neutral charge [4]. Although cysteine levels are typically much lower than those of GSH when tumour cells are grown in tissue culture [5], Koch and Evans have shown large increases in cysteine content when cells are grown as tumours in syngeneic mice [6]. Adapting the methodology used by these authors to clinical samples, we have recently found cysteine concentrations in excess of 1 m *M* in biopsies obtained from some patients with locally advanced carcinomas of the uterine cervix [7]. These findings suggest that cysteine plays a more significant role in clinical radioresistance than previously considered, and that pharmacological inhibition of tumour cysteine uptake therefore has therapeutic potential as an adjunct to radiotherapy.

GSH is a major storage and transport source of cysteine. GSH cannot be transported intact by most cells, and is broken down into its constituent moieties [8]. The membrane-bound enzyme gamma-glutamyl transpeptidase (γ-GT) is the only enzyme known to cleave GSH for cellular uptake [5]. Its action produces cysteinylglycine (*cys-gly*), and a γ-glutamyl (γ-glu) moiety that binds to acceptor molecules including cystine (*cys-cys*) [9]. Subsequently, the dipeptide *γ-glu-cys-cys* can enter the cell via amino acid transport

systems where intracellular reducing conditions favour the formation of γ-glutamylcysteine, a substrate for GSH synthetase, with the release of a cysteine molecule [10, 11]. Thus the recycling of GSH by the γ-glutamyl cycle serves as a source of intracellular cysteine.

Since GSH plays a prominent role both as an antioxidant and in resistance to cancer chemotherapy, most of the published work on γ-GT inhibition has examined the effects on cellular GSH content in relation to these actions [12, 13, 14]. To our knowledge, there have been no published studies examining the potential role for γ-GT as a source of cysteine in solid tumours. However, cellular accumulation of cysteine by the action of γ-GT has recently been shown to occur in lymphocytes, where it is suggested to protect against oxidative stress that occurs in sites of inflammation [13]. Since elevated γ-GT activity has been reported in a number of different human tumour types [15, 16, 17, 18], we examined its relation to cysteine accumulation in human cervical carcinoma cell lines maintained in tissue culture and as xenografts in immune-deprived mice, and the effects of the irreversible γ-GT inhibitor, acivicin, as a prototype cysteine-modulating agent.

Methods and materials

Reagents

Amino acids and acivicin were obtained from Sigma-Aldrich (Oakville, Ontario, Canada). Acivicin was made up as a 10 m *M* stock solution in Milli-Q water and diluted to the appropriate concentrations in physiological saline solution. All protease inhibitors and horseradish-peroxide-conjugated goat anti-rabbit IgG were purchased from VWR Canada (Mississauga, Ontario). Protein assay reagents were purchased from Pierce (Rockford, Ill.). Essentially fatty acid-free bovine serum albumin (BSA) was obtained from Boehringer Mannheim (Indianapolis, Ind.). All HPLC mobile phase reagents were obtained from Fisher Scientific (Nepean, Ontario, Canada). Fetal calf serum (FCS) was obtained from Cansera (Rexdale, Ontario, Canada).

Cell lines and in vivo tumour growth

Human squamous cell cervical carcinoma cell lines Me180, SiHa, C4I, and CaSki were obtained from the American Type Culture Collection (ATCC, Rockville, Md.). All cells were grown in alpha-minimum essential medium (α-MEM) supplemented with 10% FCS and 0.1 mg/ml kanamycin at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. All animal experiments were done in accordance with institutional guidelines for animal welfare. Me180 and CaSki cells were injected intramuscularly into the gastrocnemius muscle of female SCID mice at 6–8 weeks of age, and used when the tumour and leg diameter reached about 11 mm.

Defined media

Cysteine/cystine-free α-MEM was prepared and modified by the addition of 30 μ*M* of cysteine, 30 μ*M* of GSH or 30 μ*M* cysteine and 30 μ*M* GSH. Additions were made immediately prior to use.

HPLC measurement of intracellular cysteine

For in vitro experiments, cysteine was extracted by incubating cells in 1 ml extraction buffer. Briefly, cells were incubated in 1 ml extraction buffer (50 m *M* sulphosalicylic acid and 50 μ*M* each of EDTA, sodium diethyldithiocarbamate and diethylenetriaminepentaacetic acid) at 4°C for 10 min. Samples were then centrifuged at 16,000 *g* for 15 min to obtain visually clear supernatants which were then immediately assayed by HPLC. For tumour cysteine measurements, two 10 μm cryostat sections were cut from the frozen tissue blocks parallel to those used for the γ-GT assays, and immediately placed in 300 μl ice-cold extraction buffer for 1 h. Cysteine concentrations were calculated by comparing the area under the peak of the samples to that of known cysteine standards diluted in extraction buffer as previously described [6].

Measurement of γ-GT by biochemical assay

For in vitro experiments, cells were grown in 60 mm plastic dishes until confluent, washed twice with cold phosphate-buffered solution (PBS), then immediately incubated with 500 μl ice-cold lysis buffer (1% Triton X-100, 0.1% SDS, 50 m *M* Tris, pH 8.0, 150 m *M* NaCl, 5 μg/ml leupeptin, and 5 μg/ml aprotinin), placed on ice for 30 min and then scraped off the plates. For measurement of γ-GT in tumours, two 10 μm thick cryostat sections were cut from frozen tissue blocks, and immediately placed in 500 μl ice-cold lysis buffer. Cell lysates were then collected and centrifuged at 16,000 *g* for 15 min at 4°C. The activity of γ-GT was determined using a fluorimetric biochemical assay modified previously by Forman et al. [19]. Briefly, a 0.1 ml aliquot of the cell or tissue extract was added to a 0.25 ml reaction mixture containing 0.1 *M* 2-amino-2-methyl-1,3-propanediol (ammediol)-HCl buffer (pH 8.6), 20 m *M* glycylglycine, 20 μ*M* γ-glutamyl-7-amino-4-methylcoumarin (γ-glutamyl-AMC), and 0.1% Triton X-100. The γ-glutamyl-AMC was prepared as a 1 m *M* stock solution in methoxyethanol by sonication. The reaction took place in an incubator at 37°C for 30 min and was halted by the addition of 1.5 ml ice-cold glycine buffer (50 m *M*). Fluorescence emission of the AMC product was measured at 440 nm with an excitation wavelength of 370 nm. The concentration of protein in each sample was determined using the Bradford protein assay method [20] with BSA used as the standard, and γ-GT activity expressed as milliunits per milligram protein.

Measurement of γ-GT by antibody labelling

An anti-γ-GT antibody (GGT-129) obtained from Dr. A. Paolicchi (University of Pisa, Italy) was used to determine protein expression by immunohistochemistry. This antibody was raised against a synthetic peptide corresponding to the C-terminal 20 amino acid residues of human γ-GT heavy chain (CDTTHPISYYKPEFYTPDDGG) as previously described [14, 21]. Serial cryostat sections (thickness 5 μm) of tumour tissue were air-dried, then treated with a 0.3% hydrogen peroxide solution for 10 min followed by a multispecies blocking reagent (Dako, Mississauga, Ontario) for 20 min. Slides were incubated with the GGT129 (1:8000) overnight at 4°C, then washed and incubated with a biotinylated goat anti-rabbit IgG, followed by linker and treatment with 3-amino-9-ethyl carbazole (AEC) for 5 min. Sections were counterstained with hematoxylin, and mounted in Crystal Mount. SCID mouse kidney was used as a positive control. For immunofluorescence of γ-GT, sections were incubated GGT129 (1:8000) and with a Cy3-conjugated goat anti-rabbit secondary antibody (1:200).

Digital image analysis

Cryostat sections cut parallel to those used for γ-GT and cysteine measurements were stained with hematoxylin and eosin (H&E), and imaged using a MicroComputer Image Device (MCID; Imaging Research, St. Catherine's, Ontario, Canada) as previously described [7]. Using the MCID software program, the pixel area of the tissue section was obtained, and converted into micrometres squared using an external calibration standard. This area was then multiplied by the section thickness to determine volume of the tissue section. Using this value, tumour cysteine measurements were converted into nanomoles per milligram tissue [7].

Statistical analysis

Statistical analyses of all data were carried out using Sigma Stat 2.0 software. Control and treatment groups were compared by analysis of variance (ANOVA) followed by Dunnett's test, while Student's *t*-test was used for comparing data between untreated and single-treatment groups, with $P \leq 0.05$ as the criterion for statistical significance. Results are reported as means ± SEM. All experiments were repeated at least three times.

Results

Tissue culture experiments

γ-GT activity and effects of acivicin in cervical carcinoma cell lines

Enzyme activities determined by the biochemical assay, and the response to the γ-GT inhibitor acivicin are illustrated in Fig. 1. The Me180 cell line showed a considerably greater amount of enzyme activity (119.89 ± 28.76 mU/mg protein) than the other three cell lines (SiHa 13.14 ± 1.28, C4I 11.69 ± 1.46, CaSki 3.55 ± 0.61 mU/mg protein). This range in activity levels is comparable to that previously reported for other human cancer cell lines [14]. Pretreatment with acivicin for 3 h resulted in a dose-dependent inhibition of γ-GT activity in Me180, SiHa, and C4I cells, with an IC_{50} value of approximately 40 μ*M*. The activity obtained for CaSki cells was at the lower limit of detection for the assay used in these experiments, and no significant decrease in activity was seen with acivicin treatment. Furthermore, whereas Western blotting using the GGT129 antibody detected a strong 66 kDa band corresponding to the heavy subunit of γ-GT in Me180 cell extracts, this was not detectable in CaSki cells (data not shown). Based on these data, Me180 and CaSki cells were selected for subsequent experiments. Cell growth was not affected since cells were nearly confluent at the time of experimentation.

Growth in thiol-modified media

Similar to most tissue culture media, α-MEM contains high levels of cysteine (200 μ*M*) compared to those found in normal human serum (approximately 30 μ*M*).

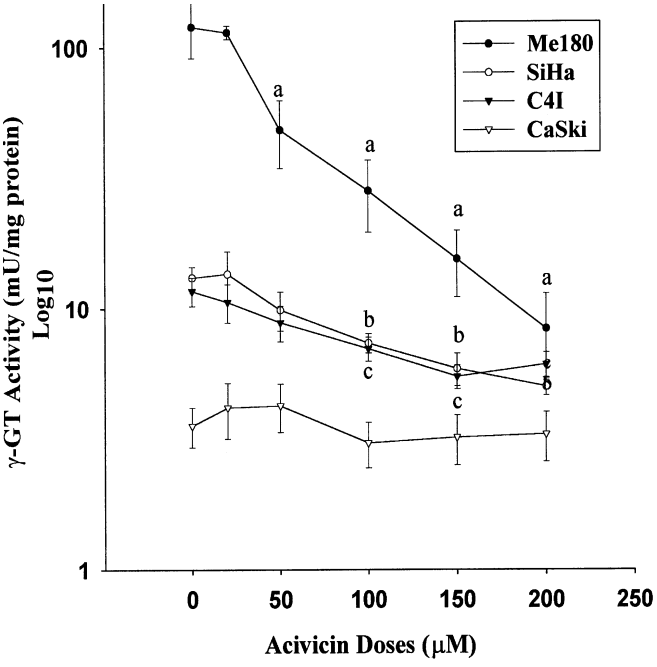

Fig. 1 γ-GT activity in cervical carcinoma cell lines using various doses of acivicin. The cells were incubated with acivicin for 3 h at 37°C prior to analysis using a fluorescence spectrophotometer. Error is expressed as SEM. Significant decreases in γ-GT activity when compared to untreated samples are signified by *a*, *b* and *c* in Me180, SiHa and C4I cells, respectively, with $P \leq 0.05$ and $n \geq 4$

The concentration of GSH, the substrate for γ-GT, is also approximately 30 μ*M* serum [22], but is likely to be much lower in growth medium containing 10% FCS. To determine the effects of extracellular NPSH on cysteine levels in vitro, and to simulate the in vivo situation, Me180 and CaSki cells were incubated in unmodified α-MEM, or in modified α-MEM (cysteine-free, or with the addition of 30 μ*M* cysteine, 30 μ*M* GSH, or 30 μ*M* cysteine and 30 μ*M* GSH) for 24 h. All growth media were supplemented with 10% FCS.

As seen in Fig. 2, a nonsignificant decrease in cysteine levels relative to those found in unmodified α-MEM, was seen in Me180 cells when grown in cysteine/cystine-depleted medium. The addition of 30 μ*M* cysteine did not significantly alter intracellular cysteine levels, whereas supplementation with 30 μ*M* GSH resulted in partial recovery of cysteine, and this was further enhanced when the cysteine/cystine-free medium was supplemented with both 30 μ*M* cysteine and 30 μ*M* GSH. In contrast, significant decreases in intracellular cysteine levels were found when CaSki cells were grown in cysteine/cystine-free medium alone or supplemented with 30 μ*M* cysteine and 30 μ*M* cysteine and 30 μ*M* GSH. These results suggest that this cell line uses amino acid transport for accumulation of intracellular cysteine. CaSki cells, which had the lowest γ-GT activity, were unable to recover intracellular cysteine in the cysteine plus GSH-supplemented medium, consistent with this enzyme playing a role in cysteine accumulation at physiological NPSH concentrations.

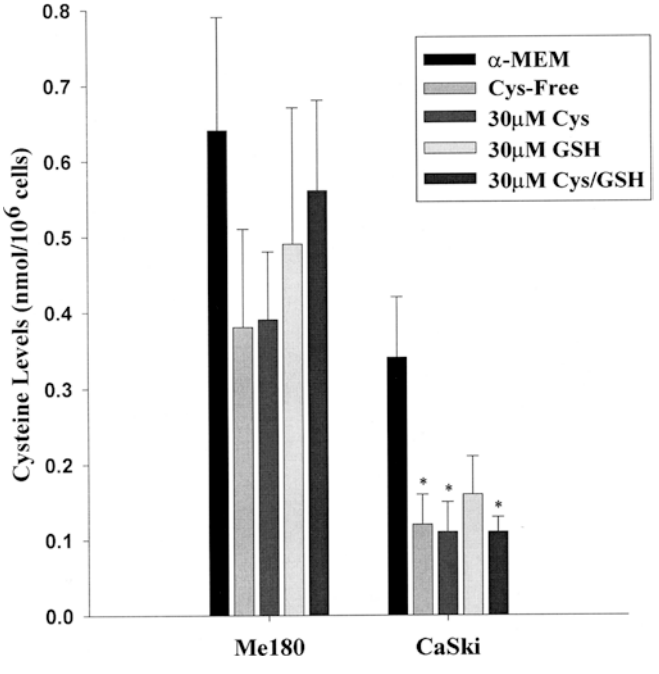

Fig. 2 The effect of varying the thiol concentrations in the growth medium on intracellular cysteine levels in Me180 and CaSki cells. Error bars are SEM. The data presented are from a minimum of three separate experiments. *$P \leq 0.05$ vs cysteine levels in α-MEM (n≥3)

Effects of acivicin on cysteine recovery from defined medium

To test if γ-GT activity was involved in the cysteine recovery by Me180 cells grown in cysteine plus GSH-supplemented medium, 200 μ*M* acivicin was added 3 h prior to HPLC analysis. Serine-borate (5–10 m *M*) was also tested as an inhibitor of γ-GT activity [23], but the decrease was not as substantial as that seen with acivicin treatment (data not shown). Thus, acivicin was used for all further experiments. As seen in Fig. 3A, acivicin treatment produced a pronounced decrease in cysteine recovery by Me180 cells. This effect was seen when the medium was supplemented with 30 μ*M* cysteine or GSH alone, probably because small amounts of GSH in the FCS were sufficient to maintain γ-GT activity. Paradoxically, acivicin treatment resulted in increases in cysteine in CaSki cells grown both in regular medium and in supplemented cysteine-free medium (Fig. 3B). These effects are likely due to additional metabolic effects of acivicin, since this agent is known to inhibit other enzymes that use glutamine as a substrate, such as those involved in nucleoside biosynthesis [24, 25]. Overall, the effects of acivicin on cysteine recovery

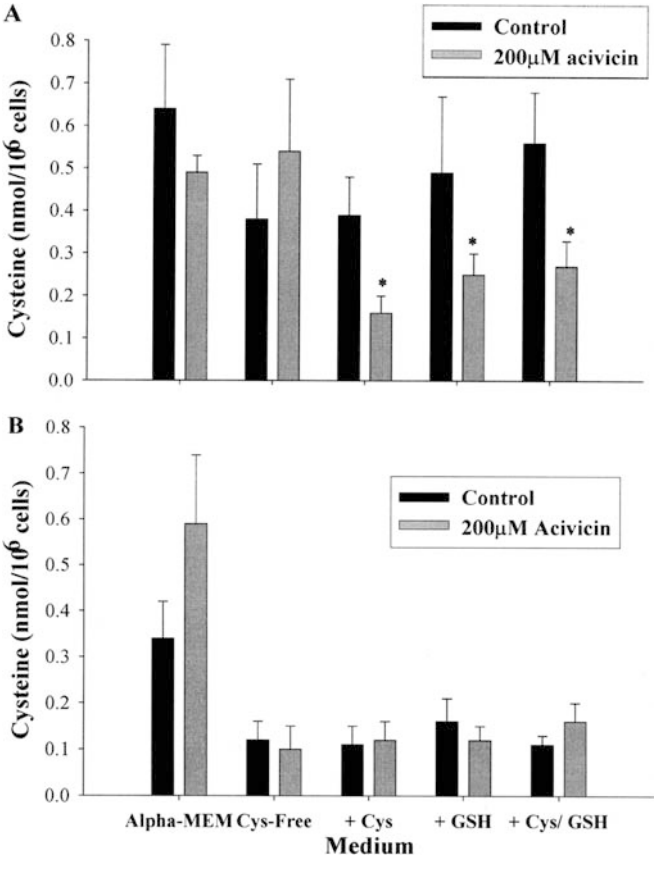

Fig. 3A, B The effect of γ-GT inhibition on intracellular cysteine levels when grown in modified medium for Me180 cells (**A**) and CaSki cells (**B**). Error bars are SEM. *$P \leq 0.05$ vs cysteine levels in untreated groups ($n \geq 7$)

from defined medium are consistent with a role for γ-GT in high-expressing Me180 cells.

In vivo experiments

Detection of γ-GT activity in xenografts

Me180 and CaSki cells grew readily as xenografts in SCID mice. Measurements of γ-GT activity were made on frozen sections cut with a cryostat, using a modification of the fluorimetric technique used for the tissue culture experiments. Parallel sections were labelled with H&E, and those containing >25% necrotic or stromal tissue were omitted from subsequent biochemical analyses. Me180 xenografts showed a mean γ-GT activity of 24.69 ± 10.40 mU/mg protein, and CaSki xenografts a mean of 4.00 ± 2.15 mU/mg protein. Using immunohistochemistry, γ-GT was readily detected in Me180 xenografts (Fig. 4A). In contrast to in vitro growth, where the protein was undetectable by Western blot (data not shown), CaSki xenografts showed weak immunohistochemical staining for γ-GT (Fig. 4B), suggesting that this protein can be upregulated in the in vivo situation.

Effects of acivicin treatment

To determine the time course for γ-GT inhibition by acivicin, Me180 xenograft-bearing mice ($n = 4$ per group) were treated with 35 mg/kg acivicin and killed at various times. The dose of acivicin used was based on a previous report [22]. As seen in Fig. 5, acivicin produced a rapid decrease of >90% in γ-GT activity that was sustained for at least 4 h, consistent with previously published data [26]. Based on these data, a 2-h treatment protocol was used for subsequent experiments. Next, an acivicin dose-response curve was obtained. Me180 and CaSki xenografts were treated with various doses of acivicin for 2 h and analysed for enzyme activity. The doses given were: 17.5, 35, 70 and 140 mg/kg. All doses of acivicin decreased γ-GT activity in both the Me180 and CaSki xenografts (data not shown). Based on these data, a dose of 35 mg/kg was selected for subsequent experiments.

Groups of Me180 and CaSki xenograft-bearing mice ($n \geq 13$) were then treated with 35 mg/kg acivicin or vehicle control for 2 h, killed, and tumour γ-GT and cysteine levels determined. As shown in Fig. 6A, a decrease in enzyme activity was seen in both tumour types to lower levels of detection (Me180 from 24.69 ± 10.40 to 1.21 ± 0.16 mU/mg protein; CaSki from 4.00 ± 2.15 to 1.87 ± 0.59 mU/mg protein). Cryostat sections cut parallel to those examined for γ-GT activity were analysed for cysteine levels using HPLC. The mean cysteine level in untreated Me180 tumours was 0.32 ± 0.06 nmol/mg tissue, similar to previously reported values [27], and 0.38 ± 0.05 nmol/mg in CaSki tumours. As seen in Fig. 6B, treatment with 35 mg/kg acivicin resulted in a decrease in cysteine levels in the Me180 tumours ($n = 17$) to 0.21 ± 0.01 nmol/mg tissue ($P \leq 0.05$) and 0.33 ± 0.08 nmol/mg in CaSki tumours ($n = 15$; P n.s.).

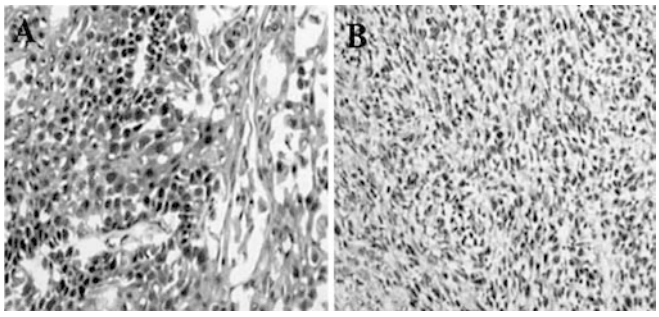

Fig. 4A, B γ-GT localization in Me180 and CaSki xenografts. The anti-γ-GT antibody was used to determine the location of γ-GT protein in the xenografts. γ-GT is shown as dark staining throughout the Me180 xenograft (**A**). The staining in the CaSki xenograft is mainly counterstain (hematoxylin) and is seen as dark staining (**B**). (×250)

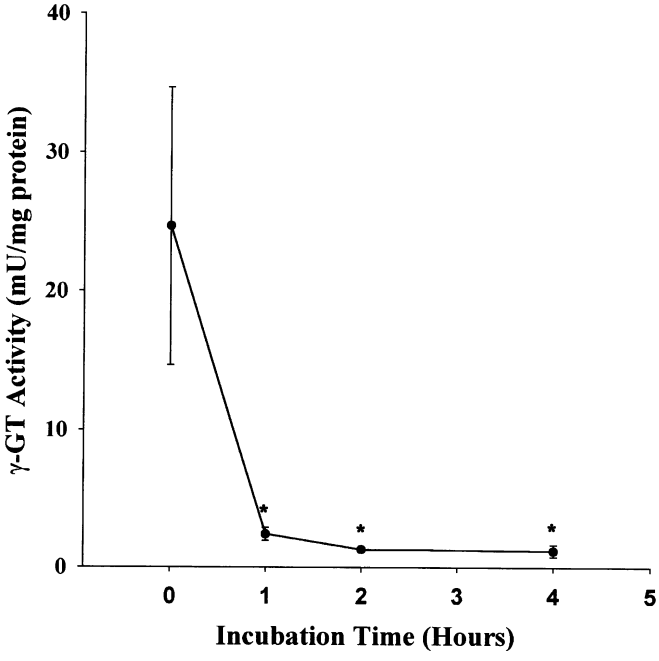

Fig. 5 Time course data for inhibition of γ-GT activity in Me180 xenografts with 35.0 mg/kg acivicin. Each time-point was repeated with four tumours. Error is expressed as SEM. *$P = 0.015$, decrease in γ-GT activity

Discussion

Radiotherapy is the mainstay of treatment for several types of cancer. Although this treatment is curative in approximately 50% of patients with locally advanced carcinomas of the uterine cervix [28], failure to control the primary tumour remains a major clinical problem. Radioresistance in cancer patients is the result of multiple mechanisms that probably interact. These

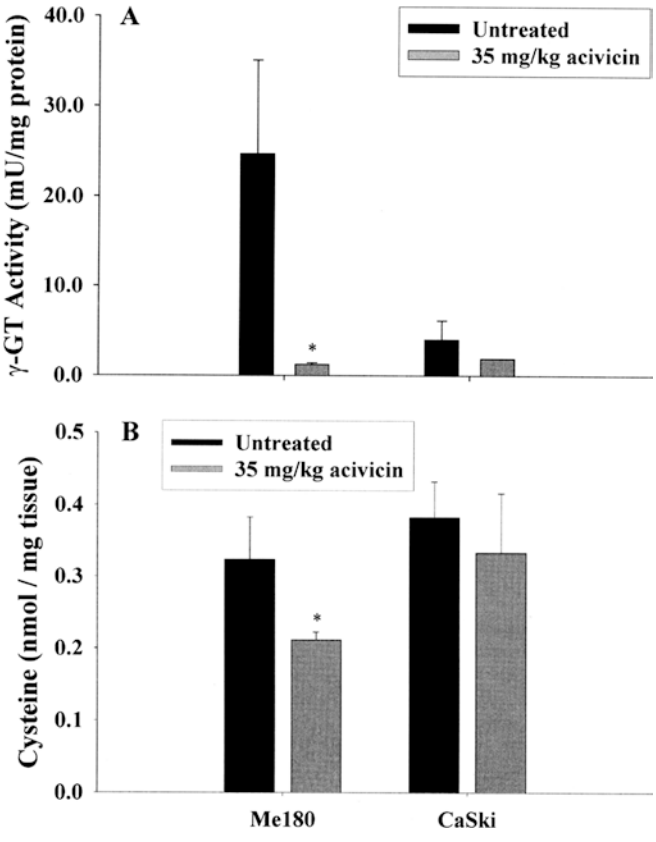

Fig. 6 **A** Effect of γ-GT inhibition with 35 mg/kg acivicin on enzyme activity in Me180 and CaSki xenografts. Enzyme levels are expressed as milliunits of γ- GT activity per milligram of protein. Error bars are SEM and $n \geq 13$. $^*P \leq 0.05$, decrease in enzyme activity. **B** Effect of γ-GT inhibition with 35 mg/kg acivicin on cysteine levels in Me180 and CaSki xenografts. Error bars are SEM and $n \geq 13$. $^*P \leq 0.05$, decrease in cysteine levels

mechanisms include enhanced DNA repair systems [29], tumour growth kinetics [30], and the presence of tumour hypoxia [1]. Additionally, NPSH including GSH and cysteine are able to chemically repair DNA radical sites produced by ionizing radiation [31]. Since NPSH and oxygen compete for DNA radicals, and oxygen fixation stabilizes DNA damage, NPSH are believed to exert greater radioprotection under hypoxic conditions that occur in human solid tumours [2].

Although GSH is less efficient than cysteine in the repair of DNA radical sites [4, 8], it is the predominant NPSH seen in tissue culture and has therefore been considered to be most relevant to radioresistance. However, tumour cells grown in syngeneic mice have been found to contain significantly greater amounts of cysteine [6] than seen during in vitro growth, indicating that in vivo growth conditions can enhance cysteine accumulation. Furthermore, in a recent study of multiple biopsies obtained from cervix cancer patients, we found that cysteine concentrations in some tumours greatly exceeded those typically seen in human cervix cancer cell lines maintained in tissue culture [7]. Cysteine might therefore be a significant determinant of radioresistance in cervix cancer patients. Hence pharmacological modulation of the underlying biochemical mechanisms has therapeutic potential.

Based on its known mechanism of action, and its overexpression in a wide range of human cancers, we investigated the effects of γ-GT on cysteine levels in human cervical carcinoma cells grown in vitro and in vivo. Enzyme activities were in the general range of those previously reported for other human tumour cells [14], with distinctly greater levels in Me180 cells than in the three other lines tested. This difference relative to low-expressing CaSki cells was maintained during growth as xenografts in SCID mice. No correlation was found between γ-GT and cysteine levels during growth in regular tissue culture medium. However, when the NPSH levels were modified to simulate more closely those found in tissue fluid, Me180 cells were able to maintain cysteine levels close to those seen in regular medium whereas CaSki cells, which had undetectable γ-GT levels, showed greatly decreased cysteine. Recovery of cysteine by Me180 cells was blunted by the γ-GT inhibitor acivicin, consistent with this enzyme playing a significant role in cysteine metabolism in vitro. Treatment with acivicin at dose schedules that profoundly inhibited γ-GT in vivo resulted in significant cysteine depletion in Me180 xenografts, but not in CaSki xenografts.

Although the data reported here are consistent with γ-GT playing a role in cysteine accumulation in human cervical carcinomas, and support the further investigation of therapeutic γ-GT modulation, it is clear that γ-GT activity is not the only determinant of tumour cysteine levels since these were similar in Me180 and CaSki tumours despite the large differences in γ-GT activities. Additional factors likely to be active in vivo include the expression of amino acid transport systems by the tumour cells, and the availability of extracellular cysteine. The latter is in turn affected both by dietary cysteine, and by transsulphuration from methionine which occurs predominantly in the liver. Since the cysteine concentrations that we found in cervical carcinoma xenografts are distinctly lower than those in the cervix cancer patient samples (approximately 0.3–0.4 m *M* vs 0.34–1.40 m *M*) [7], it is possible that the relative importance of the various cysteine uptake pathways in the xenografts does not reflect the clinical situation.

To our knowledge, there are no published data examining γ-GT levels in biopsy samples obtained from cervix cancer patients, although Hanigan et al. [21] have reported positive immunohistochemical staining for γ-GT in a range of human epithelial cancers including breast and prostate cancers. Using the GGT-129 antibody, we have found expression levels similar to those seen in Me180 in a limited number of patient samples. However, at the present time it is not known if γ-GT expression in cervix cancer patients correlates with

NPSH levels, as predicted by the results reported in the present paper.

Since cysteine protects cells from the lethal effects of ionizing radiation, and the levels in some human cancers greatly exceed those found in normal tissues, pharmacological modulation of the biochemical pathways involved in tumour cysteine accumulation would be of considerable interest in the clinic. In the study reported here we showed that treatment with the γ-GT inhibitor acivicin significantly decreased cysteine levels in Me180 xenografts consistent with this effect in vitro. Although acivicin appears to be the most effective γ-GT inhibitor currently available for in vivo use, the achievable blood concentrations in humans are only in the range 1–2 μM due to central nervous system toxicity [32], and it is therefore unlikely that acivicin could be successfully used as a γ-GT inhibitor to treat cancer patients. However, recent progress in the area of rational drug design has greatly facilitated the development of selective enzyme inhibitors, and will potentially result in effective γ-GT inhibitors becoming available for clinical trial. Meanwhile, further research is needed to elucidate the mechanisms of cysteine accumulation in human solid tumours, and the effects on cancer therapy.

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