Abstract. Background: Glutaminase activity is correlated with cancer proliferation and with growth rate in normal cells. Ehrlich ascites tumour cells (EATC) and their derivative 0.28AS-2 cells, which express antisense glutaminase mRNA, show differences in both morphology and tumorigenic capacity. Materials and Methods: Cell viability was determined with the microtetrazolium cytotoxicity test assay. Immunofluorescence staining with annexin-V and propidium iodide was carried out to assess the number of apoptotic cells. Results: 0.28AS-2 cells are less resistant to H2O2 than EATC, since half the concentration of H2O2 caused a similar effect on the cell population in 24 h. Methotrexate significantly inhibited the proliferation of both EATC and 0.28AS-2 cells at concentrations higher than 64 nM after 48 h of exposure. Conclusion: 0.28AS-2 cells are highly sensitised to methotrexate. These results provide insights into the possible role of glutaminase in cancer therapy by demonstrating that the expression of antisense mRNA for glutaminase decreases chemoresistance to some pro-apoptotic agents.

Phosphate-activated glutaminase (GA, EC 3.5.1.2) is a key enzyme in rapidly proliferating cells, suggesting a regulation of the host glutaminase in order to increase the circulating glutamine levels needed for tumour growth (1). Ehrlich ascites tumour cells (EATC), transfected with the pcDNA3 vector containing an antisense segment (0.28 kb) of rat kidney glutaminase, showed impairment in the growth rate and a shortage in the glutaminase protein and activity as compared with the parental cells. Furthermore, the transfected cells, named 0.28AS-2, also displayed remarkable changes in their morphology and, interestingly, they lost their tumorigenic capacity in vivo (2). On the other hand, we have recently demonstrated that antisense glutaminase inhibition decreases glutathione anti-oxidant capacity and increases apoptosis in 0.28AS-2 cells (3).

Glutamine has been associated with maintenance of bcl-2, a protein that inhibits apoptosis (4). Whereas glutamine-starved cells are more sensitive to Fas ligand-mediated apoptosis, they are desensitised against the cytotoxic effects of TNF-α (5). Since oxidative stress is closely associated with the induction of apoptosis in lymphocytes, it was found that glutamine significantly decreased reactive oxygen species levels in activated T cells. By studying normal peripheral lymphoproliferation, it was also found that the presence of glutamine increased lymphoproliferation as well as bcl-2 levels (6). Glutamine enhanced proliferation and decreased apoptosis in intestinal epithelial cells (7). Nutritional glutamine was also found to promote cell proliferation and to prevent cells from undergoing apoptosis (8). Glutamine oxidation, by causing a change in the glutathione redox status within tumour mitochondria, activates the molecular mechanism of apoptotic cell death (9). Glutamine via glutamate is one of the precursors for the synthesis of glutathione (GSH), the major endogenous anti-oxidant in mammalian cells, which protects cells from oxidative injury and death. Cancer cells have higher GSH levels than the surrounding normal cells, which attributes to a higher rate of cell proliferation and resistance to chemotherapy. Therefore, the selective tumour depletion of GSH presents a promising strategy in cancer treatment. Experimental studies have associated decreased GSH levels with the inhibition of proliferation and the stimulation of apoptosis (10).
0.28AS-2 cells, with reduced glutaminase activity in relation to that of the control EATC, were used in this study as a model. Methotrexate (MTX) and hydrogen peroxide were used to induce apoptosis, and these chemicals were assayed to better understand the molecular basis of their toxicity in relation to glutaminase inhibition. Methotrexate is widely used for cancer treatment, and has demonstrated consistent activity against several malignant tumours (11). The novel aspect of this work was to analyse the sensitisation of tumour cells to methotrexate as well as the levels of apoptosis in response to drugs employed in cancer therapy. We hypothesised that the use of chemotherapeutic agents combined with glutaminase inhibition results in enhanced apoptosis and decreased methotrexate toxicity, leading to relief of both disease and symptoms.

Materials and Methods

Cells and culture. EATC (ATCC, Manassas, VA, USA) and its derivative 0.28AS-2 were grown in RPMI (Sigma, St. Louis, MO, USA) medium supplemented with 10% FCS and 100 units/mL penicillin, 100 mg/mL streptomycin and 1.25 mg/mL amphotericin (BioWhittaker, Walkersville, MD, USA). Cultures were incubated at 37°C with 5% CO₂/95% air. 0.28AS-2 cells were obtained by EATC lipofection (2).

Cell growth and viability. Cell enumeration was carried out with a haemocytometer and a ZM Counter (Coulter, Luton, UK). Before apoptosis evaluations, IC₅₀ values were determined in clonogenic survival assays of EATC and 0.28AS-2 cells for MTX and H₂O₂ at various concentrations. In order to characterise the time-course action of these chemicals on cell proliferation, cell viability was examined by the MTT (microtetrazolium) cytotoxicity test assay. Cells were seeded at a density of 20,000 cells/mL in 96-well culture plates. After 24-h incubation, the cells were rinsed with PBS and further incubated with fresh medium containing MTX or H₂O₂. Finally, the cells were treated for 24, 48, 72 or 96 h, and cell viability was assayed by the MTT method. Briefly, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added at a concentration of 0.5 mg/mL and incubated at 37°C in a CO₂ incubator for 3 h. One hundred µL/well 0.04 M HCl in 2-propanol was directly added into the wells. After 30 min, the absorbance was measured at 570 nm by use of a ELISA microplate reader and analysed using SOFTmax PRO software (Molecular Devices, Sunnyvale, CA, USA).

Phosphatidylserine membrane asymmetry assay. For annexin-V detection, 500,000 cells were incubated with the respective chemicals, harvested, washed with PBS and then suspended in binding buffer. The fluorochrome fluorescein isothiocyanate (FITC) conjugated to annexin-V, a 35-36 kDa calcium-dependent phospholipid-binding protein with high affinity for phosphatidylserine, gives off a characteristic green fluorescence when excited under blue light. Double immunofluorescence staining with annexin-FITC and PI introduces values for a convenient assessment of absolute cell counts on double platforms. Cells were stained with FITC-labelled annexin-V, as described by the manufacturer (annexin-V-FITC assay kit purchased from MBL, Nagoya, Japan), and 10 µL of a stock solution of propidium iodide (PI, 20 µg/mL). Flow cytometric determinations were performed, as previously described (3). Log FL1, Log FL2 and Log FL3 dot blots were used to distinguish live, apoptotic and necrotic cells, based upon the simultaneous determination of the translocation of phosphatidylserine and the changes in plasma membrane integrity that accompany apoptosis. In this way, necrotic cells PI-positive for both FL2 and FL3 were excluded from further analysis. Apoptotic cells are positive for annexin-FITC (12). Apoptotic subpopulations can be quantified as shown in Figure 1 (13).

Statistical analysis. In the MTT assay, the results are expressed as the mean and standard deviation (SD) from three independent
values. The statistical significance of experimental data was evaluated by the Mann-Whitney U-test. $p<0.05$ was considered as statistically significant.

**Results**

*Dose and time rate effect of hydrogen peroxide and methotrexate on cell viability.* In order to analyse the biological effect associated with the down-regulation of glutaminase expression, the growth of EATC and 0.28AS-2 cells on treatment with H$_2$O$_2$ and MTX was meticulously determined by the MTT assay. Chemicals were administered to the cells 24 h after plating, and the effects of H$_2$O$_2$ and MTX concentration were examined after 24, 48, 72 and 96-h exposure, respectively. As shown by the MTT assay, the long-term action of both molecules (up to 48 h) caused a dose-dependent loss of viability (Figures 2 and Figure 3). EATC treatment with a concentration higher than 50 ÌM H$_2$O$_2$ resulted in the destruction of the population in 24 h (Figure 2A). 0.28AS-2 cells were less resistant to H$_2$O$_2$ than EATC, since half the concentration of H$_2$O$_2$ (25 ÌM) caused a similar effect on the cell population in 24 h (Figure 2B). Methotrexate significantly inhibited the proliferation of both EATC and 0.28AS-2 cells at concentrations higher than 64 nM after 48 h of exposure (Figure 3). This effect was also observed at lower concentrations of the drug (16 nM, 32 nM and 64 nM) on the 0.28AS-2 cells (Figure 3B). The action of MTX in 0.28AS-2 cells over long incubation times, even at low concentrations, is noteworthy. These findings are strongly in agreement with the drug sensitisation of the cell line expressing antisense glutaminase.

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**Figure 2.** Effect of H$_2$O$_2$ on the viability of EATC (A) and 0.28AS-2 cells (B). 0.28AS-2 cells were only slightly more sensitised than the EATC, with a significant difference in viability only at 12.5 and 25 ÌM H$_2$O$_2$. Cells were seeded at a density of 2x10$^4$ cells/mL in a 96-well culture plate and treated for 24, 48, 72 or 96-h culture. Finally, MTT was added at a concentration of 0.5 mg/mL and incubated for 3 h as detailed in the Materials and Methods section. After 30 min, the absorbance of sample was measured at 570 nm, and results are depicted in the figure. Each set of bars represents a growing curve for each H$_2$O$_2$ concentration. The control (Ctl) bar represents absorbance at zero time in the absence of any drug treatment. Results are means for at least three different wells and a representative of at least three individual experiments, with a standard deviation (SD) less than 10%. 

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Figure 3. Effect of methotrexate on the viability of EATC (A) and 0.28AS-2 cells (B). 0.28AS-2 cells treated with MTX had an increased ratio of apoptosis compared to EATC. Cells were seeded at a density of 2x10^4 cells/mL in 96-well culture plate, and treated for 24, 48, 72 or 96-h culture. Finally, MTT was added at a concentration of 0.5 mg/mL and incubated for 3 h as detailed in the Materials and Methods section. After 30 min, the absorbance of the sample was measured at 570 nm, and the results are depicted in the figure. Each set of bars represents a growing curve for each methotrexate concentration. The control (Ctl) bar represents absorbance at zero time in the absence of any drug treatment. The results are means for at least three different wells and are representative for at least three individual experiments, with a standard deviation (SD) less than 10%.

Figure 4. IC_{50} values in EATC (A) and 0.28AS-2 (B) cells for H_2O_2 and methotrexate. The results are means for at least three different wells and are representative of at least three individual experiments, with a standard deviation (SD) less than 10%.
As illustrated in Figure 4A, 72 h after H₂O₂-treatment, EATC and 0.28AS-2 cells showed time-dependent reduced cell growth, with IC₅₀ values of 23 and 22 μM, respectively. Although 0.28AS-2 cells were more sensitised to H₂O₂ than EATC (Figure 2), the MTX effect was even more important (Figure 3). In fact, 72 h after the presence of MTX, the growth of both the EATC and 0.28AS-2 cells was also reduced in a time-dependent manner, and IC₅₀ values of 52 and 12 nM, respectively, were obtained (Figure 4). It must also be emphasised that basal absorbance levels at 570 nm (time zero in the absence of any drug treatment) for EATC (Figures 2A and 3A) were double the values of the 0.28AS-2 cells (Figures 2B and 3B).

Apoptosis assays in response to hydrogen peroxide and methotrexate action. Annexin-V binding assays were used to detect the loss of phospholipid membrane asymmetry and exposure of phosphatidyserine at the cell surface, an early event in the sequence leading to apoptotic cell death (Figure 1). These experiments showed that 32 nM of MTX did not affect the relative number of apoptotic cells in EATC, but enhanced the ratio of apoptosis in 0.28AS-2 cells (p<0.01). This effect was even augmented at 64 nM MTX when 4 times as many 0.28AS-2 cells were apoptotic compared to the control EATC (p<0.01). Therefore, EATC are more resistant to MTX-induced apoptosis than 0.28AS-2 cells. On the other hand, basal levels (at zero time and in the absence of any added chemical) of apoptotic 0.28AS-2 cells are higher than those of the control EATC. It can thus be stated that antisense glutaminase expression induces apoptosis and seriously sensitises Ehrlich tumour cells to MTX action.

Discussion

This work corroborates the very recent evidence provided by antisense technology for inducing apoptosis (14), inhibiting cell proliferation (15) and using therapeutic antisense for enhanced chemotherapy sensitisation of cancer cells (16). In a previous work, we demonstrated that epithelial mucin-1 (MUC1), a protein involved in the immune defense mechanism against cancer, was markedly diminished in 0.28AS-2 cells (17). The MUC1 is aberrantly overexpressed by most human carcinomas. Recent studies demonstrated that MUC1 attenuates oxidative stress and sensitises cells to H₂O₂-induced cell death. These findings indicate that MUC1 regulates the signalling pathway in a survival response to oxidative stress (18). Therefore, reduced MUC1 expression in 0.28AS-2 cells fits with our model, which demonstrated an activation of apoptosis in cells with decreased glutaminase expression.

Two major apoptosis signalling pathways are known: the mitochondrial pathway (19) and the death receptor pathway (20). Further research is needed to ascertain if antisense glutaminase expression involves activation of the mitochondrial or death receptor signalling pathway. It is known that antisense oligonucleotides, which down-regulate bcl-2 or bcl-xL expression, induce apoptosis and synergistically interact with chemotherapy (21). In previous studies it has also been shown that exposure of human cells to bcl-xL antisense oligonucleotides sensitises human cancer cells to conventional chemotherapeutic agents (22). Similar approaches, using a combination of molecular and conventional treatment, may have clinical utility for other tumours (23). In any case, since the glutaminase antisense approach has the potential to facilitate apoptosis, the use of this technique in combination with others merits in vivo testing in cancer models.

It has been previously stated that glutamine both facilitates chemotherapy while reducing toxicity (24), and increases methotrexate concentrations in tumour tissues (25). Our study documented the changes in chemoresistance to MTX in the glutamine-enriched 0.28AS-2 cells, in comparison with EATC, thereby supporting previous results indicating sensitisation of tumour cells to the cytostatic agent methotrexate through the inhibition of some overexpressed genes (26).

Our results can be exploited to gain new insights into the underlying biological basis for the connection between the antiproliferative activity of specific antisense expression and the promotion of apoptosis (27), and cancer therapy (28). In addition, glutaminase inhibition and glutamine supplementation (29) can be useful tools to increase the comfort and health of many patients receiving intensive cancer chemotherapy.

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References


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