Cultured human cells can acquire resistance to the antiproliferative effect of sodium aurothiomalate

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SUMMARY Cultured human epithelial cells (HE), grown as monolayers, acquired resistance to otherwise lethal concentrations (300 µmol/l, culture medium) of sodium aurothiomalate during five months' exposure to stepwise increased concentrations of the drug. The resistance acquired was shown by exposure to drug concentrations ranging from 25 to 300 µmol/l, resulting in 100% of the resistant cells (HE_{Myo}) surviving compared with controls. Only 13% of the sensitive parent cells survived when exposed to 300 µmol/l for four days. The HE_{Myo} cells were also resistant to the antiproliferative effects of equimolar concentrations of thiomalic acid without gold. The cytosolic gold concentration and the association of ¹⁹⁹Au with cytosolic proteins after gel filtration were similar in both cell lines after sodium aurothiomalate exposure to the exponentially growing cells. No synthesis of gold binding proteins of metallothionein character was observed in the HE_{Myo} cells. The concentration of free thiomalate in the sonicates and cytosols of the HE_{Myo} cells was decreased to 25–30% of the concentration found in the HE cells. Comparison with previous data for the cytosolic concentration of total thiomalate in the HE cells suggests that most of the cytosolic thiomalate present was free thiomalate. We conclude that the cells can develop resistance to the antiproliferative effect of sodium aurothiomalate, and that the resistance may be due to their capacity to maintain low concentrations of free thiomalate in the sonicates and cytosols. The results support previous findings that sodium aurothiomalate appears to dissociate within cells.

Key words: cells—cultured, drug resistance, thiomalate.

It is a well known phenomenon that drugs containing gold fail to induce remission in all patients treated for rheumatoid arthritis.^{1–3} The percentage of non-responders ranges from 5 to 50% depending upon the criteria used for defining response. Furthermore Evers and Sundstrom have shown that the percentage of non-responders to sodium aurothiomalate treatment increases during a second course of therapy.⁴ Only four of 11 patients who developed complete remission during the first course of therapy sustained complete remission with

the second course, and none of the 12 initially non-responders or partial responders improved with the second course of therapy. The mechanisms of this failure are not known, but one explanation could be acquired drug resistance.

We have previously developed two substrains of cultured human epithelial cells which are resistant to otherwise lethal concentrations (2 µmol/l) of auranofin, a drug containing gold (I).⁵ One substrain (HE₁₀₀) was originally made resistant to 100 µmol/l of cadmium chloride⁶ and contained high amounts of cytosolic metallothionein (MT), a metal binding protein induced by cadmium pretreatment. The demonstrated binding of gold by MT is one possible mechanism of auranofin resistance in these cells. On sodium aurothiomalate exposure (25–300 µmol/l),

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however, no differences in sensitivity to the growth inhibitory effect were observed between the HE₁₀₀ cells and the parent cell line.⁷ Another substrain (HE_{AF}) developed resistance to auranofin during exposure to stepwise increased concentrations of the drug in the culture medium. In these cells cytosolic gold was bound by low molecular weight gold binding proteins appearing on auranofin treatment. Furthermore, the HE_{AF} cells had the capacity to depress the cellular gold concentration.

The present study was undertaken to investigate whether the parent line of human epithelial cells could also acquire resistance to the antiproliferative effect of sodium aurothiomalate during exposure to stepwise increasing concentrations of the drug. We also investigated possible mechanisms for the development of resistance, including MT synthesis induced by sodium aurothiomalate, and alterations in cellular concentrations of gold and free thiomalate.

Materials and methods

THE CELLS

Human epithelial cells (HE) derived from normal skin (NCTC 2544) and purchased from the American type culture collection, Maryland, USA were grown in monolayer cultures as previously described.⁸ The cells have been characterised as human and epithelial by surface antigen typing, isoenzyme determination, and transmission electron microscopy.⁹ A substrain (HE_{Myo}) has been made resistant to the antiproliferative effect of 300 µmol sodium aurothiomalate/l culture medium, and grown for months with this otherwise lethal concentration of the drug (see below).

DRUGS

The sodium aurothiomalate was the commercially available drug, Myocrisin, Sodium [¹⁹⁹Au]aurothiomalate (specific activity 163 mCi/mmol) was synthesised at the Department of Biophysics, University of Manchester, England. Thiomalic acid was purchased from Sigma Chemical Co, Mo, USA. All compounds were stored at 4°C and protected from light. The thiomalic acid was kept under an atmosphere of nitrogen. Fresh drug solutions were prepared for each experiment.

DEVELOPMENT OF SODIUM AUROTHIOMALATE RESISTANT CELLS

The parent HE cells were exposed to stepwise increasing concentrations of sodium aurothiomalate, starting with 100 µmol/l culture medium and increasing by 25–50 µmol/l steps up to a final

concentration of 300 µmol/l. The intervals between each increase were about four weeks.

DRUG SENSITIVITY STUDIES

HE and HE_{Myo} cells were subcultured into Costar tissue culture wells (16 mm), at a density of 1.0×10^4 cells/well. After 24 h omission of sodium aurothiomalate both cell lines were exposed to 25, 50, 100, 150, or 300 µmol/l culture medium of sodium aurothiomalate or thiomalate derived from thiomalic acid for four days. Controls were grown without drug supplements. Cells from six replicate wells of each cell line, drug, and concentration were harvested by trypsinisation and counted in a Coulter counter at the start and on each day during the experiment. Differences in sensitivity to the drugs between the cell lines were calculated by means of an area method.⁷ The viability of the cells at the end of the experiment was determined by the dye exclusion test (trypan blue).¹⁰

CELLULAR CONCENTRATIONS AND DISTRIBUTION OF Au AND FREE THIOMALATE

Exponentially growing HE and HE_{Myo} cells were cultured without the drug supplement for 24 h and then exposed to 300 µmol/l of sodium aurothiomalate for another 24 h. After harvesting of the cells, sonicates were obtained by ultrasound sonication (15 s × 4, intervals 1 minute, on ice). Cytosols were obtained by subsequent ultracentrifugation (105 000 g, one hour, 4°C). Aliquots of sonicates and cytosols were removed for determination of the concentration of Au (by flame atomic absorption spectrophotometry) and protein (Abbott bichromatic analyser 100, with human albumin as a standard). The free thiomalate, i.e., the thiol, in sonicates and cytosols was separated by reverse phase high performance liquid chromatography (HPLC) and detected with a gold electrochemical cell, according to the method described by Rudge et al for thiomalate in plasma.¹¹ The samples for HPLC were immediately transferred to ice cold tubes containing sodium ethylenediaminetetraacetate (final concentration 5 mmol/l), and the proteins were precipitated by the addition of 400 µl of 1 M HClO₄ to 1 ml of sonicate or cytosol. After centrifugation (9000 g, five minutes, 4°C, Beckman microfuge B) the pH in the supernatant was adjusted to 5.0 by addition of K₂CO₃. Samples were flushed with nitrogen and stored at 4°C for a maximum of three hours before the HPLC separation. Differences in concentrations and distribution of Au and free thiomalate were tested by Student's *t* test, with *p* values < 0.05 being considered significant.

Determination of the cytosolic distribution of ¹⁹⁹Au was carried out in a second, but similar

experiment. The cells were exposed to 300 μmol sodium [^{199}Au]aurothiomalate/l for 24 h. The cytosols obtained were applied to a Sephadex G75 gel filtration column (1.6 $\times$ 90 cm, 10 mM ammonium formate buffer as eluant, pH 8, elution rate 18 ml/h, fraction volume 3 ml). The concentration of ^{199}Au in the eluted fractions was determined with a gamma scintillation spectrometer (Packard 5221). The coefficients of variation (standard deviation as a percentage of net counts) of the counts per minute ranged between 0.2 and 18.5% for the peaks and 3.3 and 172.5% for the lowest regions between the peaks. The highest coefficients of variation did not weaken the conclusions drawn. The concentrations of Zn and Cu in the fractions were determined by flame atomic absorption spectrophotometry.

Results

ACQUIRED CELLULAR RESISTANCE TO SODIUM AUROTHIOMALATE

Most of the HE cells detached and died when grown with 100 $\mu\text{mol/l}$ of sodium aurothiomalate. The surviving cells initially altered their shape in that they became round or developed elongated cytoplasmic protrusions. A temporary growth arrest was also observed. Their appearance normalised within a few weeks, and the growth rate gradually increased. These events were observed for each increase of the drug concentration. After about five months we obtained a substrain (HE_{Myo}) of the cells which continued to proliferate in the presence of

300 μmol sodium aurothiomalate/l culture medium. This is a lethal concentration of sodium aurothiomalate for the HE cells not previously exposed to the drug. The doubling time of the HE_{Myo} cells when cultured with 300 $\mu\text{mol/l}$ of the drug was 66 h, whereas that of HE cells grown without the drug supplement was about 30 h.

EFFECTS OF SODIUM AUROTHIOMALATE AND THIOMALATE DERIVED FROM THIOMALIC ACID ON CELL GROWTH

Figs 1 and 2 show the significant difference in the response to both compounds for the two cell lines HE and HE_{Myo}. Within the concentration range of 25–300 μmol sodium aurothiomalate or thiomalate/l culture medium both compounds exert a concentration dependent antiproliferative effect on HE cells, whereas the growth of the HE_{Myo} cells apparently was not influenced by these drug concentrations. On day four of exposure to 300 μmol sodium aurothiomalate/l 13% (percentage of median values for cell counts) of the HE cells remained adherent to the wells compared with their controls, whereas 100% of the HE_{Myo} cells remained attached. When grown in the presence of an equimolar concentration of thiomalate 2% of the HE and 87% of the HE_{Myo} cells remained attached on day 4. At that point the viability of the remaining cells from either strain was above 85%. When the HE cells were cultured further with concentrations of the compounds higher than 100 $\mu\text{mol/l}$ all cells detached and died within about one week.

Fig. 1 Growth of HE and HE_{Myo} cells during exposure to 25 (●—●), 50 (○—○), 100 (■—■), 150 (▲—▲), and 300 (△—△) μmol sodium aurothiomalate/l culture medium. Cells grown without the drug supplement served as controls (■—■). △ indicates medium change. Each point is the median of six replicates. Bars represent the 25th and 75th percentiles for the distribution of cell counts.

Fig. 2 Growth of HE and HE_{Myo} cells during exposure to 25 (●—●), 50 (○—○), 100 (□—□), 150 (▲—▲), and 300 (△—△) μmol thiomalic acid/l culture medium, and of control cells (■—■) grown without the drug supplement. △ indicates medium change. Each point is the median of six replicates. Bars represent the 25th and 75th percentiles for the distribution of cell counts.

CONCENTRATIONS OF Au AND FREE THIOMALATE IN SONICATES AND CYTOSOLS

The concentrations of Au and free thiomalate in the sonicates and cytosols after 24 h exposure of exponentially growing HE and HE_{Myo} cells to 300 μmol sodium aurothiomalate/l are shown in Table 1. The concentration of Au in the sonicates of the resistant HE_{Myo} cells (12.6 nmol/mg protein) was significantly higher than that in the sensitive HE cells (4.3 nmol/mg protein), whereas no differences were found in the cytosol concentrations (8.1 and 5.0 nmol/mg protein respectively). In the HE_{Myo} cells 33% of the amount of Au in the sonicates was recovered in the cytosols compared with 53% in the HE cells. The concentrations of free thiomalate in

HE_{Myo} sonicates (3.3 nmol/mg protein) and cytosols (6.2 nmol/mg protein) were significantly lower than the concentration in HE sonicates (10.2 nmol/mg protein) and cytosols (24.7 nmol/mg protein). The cytosolic fraction of the free thiomalate was about 100% of that in the sonicates in both lines. In the cytosols of HE_{Myo} cells Au and free thiomalate were recovered in equimolar concentrations, whereas in the sonicates the molar Au concentration exceeded that of free thiomalate. In both the sonicates and the cytosols of HE cells the molar concentration of free thiomalate was higher than that of Au.

CYTOSOLIC DISTRIBUTION OF ¹⁹⁹Au

After 24 h treatment of exponentially growing HE

Table 1 Concentrations (nmol/mg protein) of Au and free thiomalate in sonicates and cytosols of HE and HE_{Myo} cells after exposure to sodium aurothiomalate.

	HE cells			HE _{Myo} cells		
	Sonicate	Cytosol	Percentage of total in cytosol	Sonicate	Cytosol	Percentage of total in cytosol
Au (nmol/mg protein)	4.3±2.6	5.0±3.7	53	12.6±3.6	8.1±6.0	33
Free thiomalate (nmol/mg protein)	10.2±4.2	24.7±18.8	110	3.3±0.2	6.2±0.8	96
Protein content (mg)	25.2±14.0	11.4±7.3		46.0±20.9	23.5±16.8	

Values are means±SD from four experiments, and the percentages are calculated from the mean values.

Fig. 3 Elution profile of ^{199}Au (—), Zn (---), and Cu (.....) after Sephadex G75 gel filtration of HE and HE_{Myo} cytosols and after treatment of the cells with sodium [^{199}Au]aurothiomalate. For details see 'Methods' and 'Results' sections.

and HE_{Myo} cells with sodium [^{199}Au]aurothiomalate, the ^{199}Au was associated with cytosolic proteins as shown in Fig. 3. The pattern of distribution was the same in both lines. ^{199}Au eluted with two peaks; about 70% eluted with void volume proteins (peak I, $V_e/V_o=1$, mol. wt $> 75\,000$) and about 10% with substances of molecular weights less than 1000 (peak II, $V_e/V_o \approx 2.5-3.0$). Cytosolic Zn and Cu peaked with the same fractions. No peaks of metals were observed with protein classes of molecular weights about 10 000, i.e., with proteins of metallothionein character.

Discussion

This study shows that cells can acquire resistance to the antiproliferative effect of 25–300 $\mu\text{mol/l}$ of sodium aurothiomalate during exposure to gradually increasing concentrations of the drug. This concentration range includes gold concentrations corresponding to those achieved in serum when the drug

is used clinically.^{12 13} The plasma levels of free thiomalate are, however, reported to be lower, namely 200–600 nmol/l (after 20 mg sodium aurothiomalate as one single intramuscular injection).¹⁴ We have previously shown that the same HE parent cell line can also develop resistance to otherwise lethal concentrations of gold(III) chloride (350 $\mu\text{mol/l}$)¹⁵ and auranofin (2 $\mu\text{mol/l}$).⁵ The cellular mechanisms of the observed resistance are apparently different, involving possible alterations of cellular targets for the gold (gold(III) chloride), the cells' ability to maintain a low cellular gold concentration (auranofin), and binding of gold by cytosolic low molecular weight proteins of possible MT nature, occurring on auranofin treatment. Evidence was obtained from the HE_{Myo} cells indicating that the sonicate, but not the cytosolic, gold concentration was significantly higher than that in the sensitive cells, thus indicating that the HE_{Myo} cells can, to some extent, keep the cytosolic gold concentration at a low level. No synthesis of

cytosolic gold binding proteins of metallothionein character was observed. The latter finding may support the suggestions of Mogilnicka and Webb that the appearance of proteins of the MT type observed in rat kidneys on in vivo treatment with sodium aurothiomalate is mediated via alterations in Zn or Cu metabolism caused by gold treatment.¹⁶ Such indirect mechanisms may be involved in intact organisms but not in isolated cultured cells.

Furthermore, the sonicate and cytosolic concentrations of free thiomalate were significantly lower in the HE_{Myo} cells than in the HE cells. This factor is probably of importance for the resistance observed, and is in accordance with the finding that HE_{Myo} cells are also resistant to the growth inhibitory effect of thiomalate derived from thiomalic acid. In a clinical study Rudge *et al* failed to show any relation between free thiomalate levels in plasma or urinary excretion of free thiomalate and the response to, or toxicity from, treatment with disodium aurothiomalate.¹⁷ Our results support their assumption that cellular levels of free thiomalate may be more relevant to the pharmacological effects of the sodium aurothiomalate than plasma levels.

The clinical studies by Jellum and Munthe¹⁸ and Rudge *et al*¹⁷ have also produced evidence for the dissociation of the gold and the thiomalate moieties of sodium aurothiomalate in serum and urine. The present results indicate that gold and thiomalate distribute differently within cells. This is in agreement with results recently obtained when HE cells were exposed to sodium [¹⁹⁹Au]aurothiomalate and disodium auro[2,3-¹⁴C₂]thiomalate.⁷ That study showed, in addition, that gold was mainly protein bound in the cytosol, whereas the thiomalate moiety was free or associated with molecules of molecular weights less than 1000. A similar dissociation of the drug was also shown in the culture medium. The cytosolic concentration of the isotopically labelled thiomalate in HE cells after a 24 h exposure to 300 µmol disodium auro[2,3-¹⁴C₂]thiomalate was 18.0±6.4 nmol/mg protein, compared with 24.7±18.8 nmol of free thiomalate /mg protein found in this study. These results suggest that a considerable fraction of cytosolic thiomalate in these cells is probably free thiomalate and not its corresponding disulphide. Furthermore, we suggest that the bulk of free thiomalate will not interact with glutathione to form mixed disulphides. The fraction of free thiomalate determined is unlikely to represent drug impurity. Rudge *et al* have found that the Myocrisin contains only 4–8% of free thiomalate.¹⁹

In conclusion, the results presented show that cultured cells can develop resistance to the antiproliferative effect of sodium aurothiomalate, and also develop thiomalate resistance. The observed resist-

ance is associated with decreased cellular and cytosolic concentrations of free thiomalate. No changes in the cytosolic concentration of gold were found. No synthesis of cytosolic MT was observed. Accumulating data indicate that sodium aurothiomalate could be composed of two active drugs, the gold and the thiomalate moiety. Further studies should focus on the pharmacology of the thiomalate moiety and its possible contribution to the beneficial and adverse effects of sodium aurothiomalate.

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