Chemical modulation of chemotherapy resistance in cultured oesophageal carcinoma cells

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Abstract
Oesophageal carcinoma is a common form of cancer in developing countries, especially in the Caspian littoral and northern China. In contrast, it has a much lower incidence in Japan, the U.S.A. and western Europe. Certainly in the case of squamous cell oesophageal carcinoma, dietary composition, smoking, alcohol and exposure to nitrosamines are major risk factors that may partly explain the disease’s geographical distribution.

The prognosis for oesophageal carcinoma is generally poor, due to the high incidence of distant metastasis and local recurrence. Combination treatment with both cisplatin and 5-fluorouracil is the most common chemotherapy regime used. We have carried out a detailed study of sensitivity of two oesophageal cell lines: OC1 cells from a squamous carcinoma of a male patient, and OC2, a squamous carcinoma obtained from a female patient. Both cell lines are sensitive to Vinca alkaloids and doxorubicin, while being quite resistant to alkylating agents such as cisplatin and 1,3-bis-(2-chloroethyl)-1-nitrosourea. This pattern of resistance suggests a possible role for glutathione S-transferase (GST) and/or glutathione (GSH) in resistance, and would seem to exclude the multidrug resistance phenotype. Both cell lines possess mainly Pi-class GSTs, and have distinct levels of GSH, with OC2 possessing some 25% of the level of OC1 cells. Effects of a variety of modulating agents on the pattern of resistance, such as the GSH depletor, buthionine sulfoximine, and the GST inhibitor, ethacrynic acid, were determined. An unexpected observation was that ethacrynic acid appears to increase the level of GSH in both cell lines.

Oesophageal cancer: incidence, prognosis and risk factors
Oesophageal cancer, although not one of the most common malignancies in western countries, remains a significant clinical condition. Within the last few decades the incidence of oesophageal adenocarcinoma has increased in western countries [1,2]. In contrast, in Japan, adenocarcinomas account for only 2% of all resected oesophageal carcinomas [3]. High incidence rates of squamous cell carcinoma continue to occur in areas of the Caspian littoral, the Transkei and northern China, with reported figures in excess of 1 per 1000 head of population, whereas elsewhere in the world, the incidence of squamous cell oesophageal carcinoma has either slightly fallen or remained static [1]. The pathology of this disease shows remarkable geographical variety, with an incidence rate of 3 per 100000 in western countries in comparison with 140 per 100000 reported in central Asia [4]. The most common pathological type is squamous cell carcinoma, although a shift to adenocarcinoma is increasingly being observed [5].

Oesophageal carcinoma is one of the most malignant tumours in humans, and is associated with poor survival rates in patients showing symptoms [6]. Carcinoma of the oesophagus arises in the mucosal layer, but soon spreads along the submucosa and through the wall of the adventitia. Because the oesophagus lacks a serosal layer, once the tumour invades the muscular layer, it can penetrate rapidly beyond the confines of the oesophageal wall to surrounding structures [1]. Spreading then also occurs rapidly to adjacent lymph nodes. The prognosis of a patient with oesophageal carcinoma is poor, owing to the frequent occurrence of both distant metastasis and local recurrence [7]. A study of survival data in several European countries between 1985–1989 has estimated an overall European 1-year relative survival rate of 33%, and a 5-year survival rate of 10% [8].

The only confirmed risk factor in adenocarcinoma of the oesophagus is Barrett’s oesophagus, a condition that is the end-result of gastro-oesophageal reflux and appears to be increasing in incidence in the western world. In contrast, there are several known risk factors associated with the development of squamous cell carcinoma of the oesophagus, the best known of which are tobacco, alcohol, nitrosamines and both mineral...
and nutritional deficiencies [1,9,10]. Alcohol and tobacco remain as being the main risk factors of squamous cell carcinoma. Aniseed aperitif, warm spirits and beer carry the highest risk. For tobacco, the risk is correlated to the duration of consumption, and decreases after quitting [2]. Salted meat, a possible source of nitrosamines, has been associated with an increased risk of 60% for oesophageal cancer [11]. Studies have also suggested that poor dietary pattern and the quality and amount of nutrient intake may be related to the development of oesophageal cancer. Elevated risks have been associated with high intake of red meat and low consumption of vegetables, fruit and legumes [11].

**Treatment of oesophageal cancer**

Surgery is considered the mainstay of therapy. However, in the majority of patients, surgical treatment is palliative, owing to the presence of advanced disease at the time of diagnosis. Overall, a 5-year survival rate of only 20% is observed after oesophageal resection [4]. It is well established that poor prognosis depends more on the biology of the tumour, the stage of the disease and the rate of local and systemic recurrence of the disease, rather than the surgical procedure [12]. Thus multimodality therapy with pre- or post-operative chemotherapy and/or radiotherapy is being extensively evaluated. Recent trials suggest that preoperative chemoradiotherapy results in improved survival compared with surgery alone [13,14]. Concurrent chemoradiation therapy followed by surgery was shown to be an effective and safe multimodal therapy for patients with primary, inoperable T4 squamous cell carcinoma of the oesophagus [15]. The rationale for this concurrent use of chemotherapy and radiotherapy is to combine an agent that has an effect upon systemic micrometastasis, with a modality that enhances local tumour control. In addition, a number of chemotherapeutic agents have radiosensitizing effects [16]. In western countries, the most standard chemotherapy protocol used to fight against oesophageal cancer involves the use of a cisplatin/5-fluorouracil combination [17]. Toxicity in chemotherapy trials is often substantial, and has tempered enthusiasm for the routine use of this treatment approach [12]. The aims of current studies are to increase further the therapeutic ratio, using refinements of radiotherapy and new chemotherapy delivery modalities [14].

Even though multi-modality treatment regimens involving the use of chemotherapy have recently begun, chemotherapy has played a minor role in the treatment of oesophageal cancer. Of the hundreds of screened cytotoxic agents, few have been tested seriously in the fight against the disease and, therefore, more effective anti-tumour agents and better combination regimens of new and available cytotoxic drugs are necessary to improve the therapeutic efficacy of multi-modality treatment [3]. In addition, further treatment strategies will also require a better understanding of the molecular biology and biochemistry of the disease, including a greater insight into the mechanism of tumour resistance to anti-cancer agents, which is a fundamental set-back in the clinical use of chemotherapy [18].

In our laboratory, the chemosensitivity of two squamous oesophageal cell lines to a range of chemotherapeutic agents was investigated. These cell lines (OC1 and OC2) were obtained from patients who had not received preoperative chemotherapy. OC1 cells were derived from malignant ascites of squamous epithelial lining of the oesophagus of a 40-year-old male Caucasian, whereas OC2 cells were obtained from a metastatic lymph node of a 65-year-old female Caucasian suffering from undifferentiated squamous carcinoma of the oesophagus [19]. Preliminary work established that these cell lines were relatively sensitive to *Vinca* alkaloids and doxorubicin, but displayed resistance to alkylating agents (Table 1). As chemotherapeutic resistance is a fundamental limitation to clinical use of chemotherapy [18], further work set out to investigate the possible biochemical mechanism of alkylating agent resistance in these cell lines.

**Chemotherapy and drug resistance**

Tumours may fail to respond to chemotherapeutic agents *de novo* and thus exhibit intrinsic or inherent resistance, or they may develop resistance to treatment after the process of an initial response of acquired resistance. An understanding of drug resistance is important in the search for new drugs, and in the design of more effective treatment regimens [18]. Various biochemical changes that are usually specific for the selecting agent characterize resistant tumour cell populations in experimental systems. These changes include: (i) alterations in drug transport, i.e. decreased drug uptake/enhanced drug transport from the cell; (ii) alterations in drug target, i.e. increased concentrations of a target enzyme or changes in affinity for an inhibitor; (iii) an increased capacity to
Table I

Estimated IC\textsubscript{50} values (\mu M) for a range of chemotherapeutic agents tested on OC1 and OC2 cells

Values shown represent the means±S.D. calculated from a minimum of \(n\) individual measurements determined from their corresponding dose-response profiles using the GraphPad Prism program. At the maximum concentration of BCNU (0.45 mM) used, OC1 cell survival decreased to \(\approx 53\%\).

<table>
<thead>
<tr>
<th>Drug</th>
<th>OC1 IC\textsubscript{50}±S.D. (\mu M)</th>
<th>n</th>
<th>OC2 IC\textsubscript{50}±S.D. (\mu M)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vinblastine</td>
<td>0.006±0.001</td>
<td>2</td>
<td>0.007±0.0003</td>
<td>2</td>
</tr>
<tr>
<td>Vincristine</td>
<td>0.015±0.005</td>
<td>3</td>
<td>0.11±0.03</td>
<td>3</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.073±0.022</td>
<td>7</td>
<td>0.048±0.01</td>
<td>7</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>0.64±0.04</td>
<td>2</td>
<td>0.16±0.01</td>
<td>2</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>4.2±1.2</td>
<td>9</td>
<td>0.72±0.17</td>
<td>10</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>4.9±0.3</td>
<td>2</td>
<td>0.18±0.02</td>
<td>2</td>
</tr>
<tr>
<td>Melphalan</td>
<td>41±12</td>
<td>12</td>
<td>3.7±1.1</td>
<td>7</td>
</tr>
<tr>
<td>Chlorambucil</td>
<td>65±8</td>
<td>9</td>
<td>14.5±4.2</td>
<td>12</td>
</tr>
<tr>
<td>BCNU</td>
<td>450±40</td>
<td>16</td>
<td>303±55</td>
<td>10</td>
</tr>
</tbody>
</table>

inactivate the drug, e.g. increased drug detoxification enzymes/increased thiol-group scavengers; (iv) a failure to engage apoptosis; and (v) a capacity to develop simultaneous resistance to more than one type of chemotherapeutic agent [the multidrug resistance (MDR) phenotype]. Classical MDR has been described as a result of overexpression of an active transport pump, P-glycoprotein, which appears to be responsible for the transport of a broad range of toxic substances, including the anti-neoplastic agents Vinca alkaloids, anthracyclines and epipodophyllotoxins, out of cells [20].

Reduced glutathione (GSH)/glutathione-S-transferase (GST)-mediated resistance to alkylating agents

Cross-resistance to diverse drugs has been associated with elevated intracellular GSH levels [21]. GSH is the most abundant non-protein thiol-containing molecule in mammalian cells (1–10 mM), and acts as a major defence mechanism against a variety of agents, including free radicals, reactive oxygen species and cytotoxic drugs [22]. GSH is generally assumed to protect cells against the cytotoxic effect of bifunctional alkylating agents by the formation of non-cytotoxic GSH adducts, a conjugation reaction that may occur spontaneously or be catalysed by the GSTs [23]. In addition, it has been shown that acquired resistance to cisplatin and alkylating agents is frequently accompanied by an elevation in intracellular GSH content [24–26]. Depletion of intracellular GSH as a strategy to increase the cytotoxic response to various chemotherapeutic agents in a variety of tumour cell lines has been the subject of a number of studies [27]. The majority of these have focused on bifunctional alkylating agents, although this approach has also proved useful in increasing the cytotoxicity of doxorubicin and platinum complexes both in cell culture and in \textit{in situ} tumours [27].

GSTs are a family of multifunctional proteins that act both as enzymes and as binding proteins in various detoxification and excretory pathways. They function primarily as phase II detoxification enzymes, and thus help to protect cells against chemical-induced toxicity and stress [28]. Both multiple cytosolic and membrane-bound GST isoenzymes exist in all eukaryotic species [29]. Cytosolic GST isoenzymes exist as homo- or hetero-dimers, whereas the microsomal forms of GST exist as trimers. Mammalian cytosolic GST isoenzymes have been assigned to the following classes: Alpha, Mu, Pi [30], Sigma [31], Theta [32], Zeta [33] and Kappa [34]. GSTs have been implicated in the development of resistance towards cytotoxic drugs, pesticides, herbicides and antibiotics. In particular, overexpression of GSTs in tumours appears to be a factor in the development of acquired resistance towards anticancer drugs [35]. Recognition that GST levels...
Intracellular GSH concentration and GST activity from cytosolic extracts of OCI and OC2 cells

<table>
<thead>
<tr>
<th></th>
<th>OCI</th>
<th>OC2</th>
</tr>
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<tbody>
<tr>
<td>GST (1-chloro 2,4-nitrobenzene) (units · mg⁻¹)</td>
<td>0.3±0.01</td>
<td>0.13±0.00</td>
</tr>
<tr>
<td>Intracellular GSH (nmol · mg⁻¹)</td>
<td>56.73±0.44</td>
<td>11.73±0.53</td>
</tr>
</tbody>
</table>

are frequently elevated in cell lines selected for resistance to anti-cancer drugs has served as a focal point to determine the role of GST in protection against such drugs [24,26,29]. Moreover, comparisons made among related breast, bladder or colon cell lines displaying intrinsic drug resistance have suggested correlation between GST expression and level of drug resistance [36–38]. Most of these resistant cell lines contain elevated levels of class Pi GST. Overexpression of both GST Alpha and Pi enzymes has also been observed both in cell lines and tumours resistant to alkylating drugs [39–43]. Consequently, GSTs have become a therapeutic target for modulation and rational drug design.

In OC1 and OC2 cells the pattern of sensitivity to Vinca alkaloids and anthracyclines appears to exclude the MDR phenotype, whereas resistance to alkylating agents suggests that the GSH/GST detoxification system may be particularly important in these cells. Immunoblot analysis indicated that in both cell lines Pi class GST was primarily observed, although some expression of class Alpha and Mu GSTs was also observed in OCI cells. Enzymic determination of GSH levels [44] also indicated that OC1 cells contained significantly higher levels of GSH than those found in OC2 cells (Table 2); OC1 cells also exhibited greater levels of GST activity than OC2 cells. A major goal of this work was to assess the contribution of the GSH/GST system to drug resistance in these cell lines through chemical modulation studies.

**Chemical modulation studies**

In order to overcome drug resistance, it is possible to 'modulate' agents implicated in resistance (e.g. levels of GSH or specific GSTs). Overall, modulation can be defined as the use of a relatively nontoxic agent, in combination with an anti-cancer drug, with an aim of improving therapeutic efficacy by subverting drug resistance mechanisms that have an adverse impact on the clinical outcome. To achieve this, tumour-specific properties involved in resistance must be targeted. However, only when the normal tissue is relatively spared will this approach be viable. The achievement of even subtle improvements in the therapeutic index may produce a significant enhancement in disease response [45]. Modulation of GSH/GSTs in drug resistance has been intensively studied. *In vitro*, preclinical and clinical studies suggest that modulation can be accomplished via alterations in GSH pools [via buthionine sulfoximine (BSO) or GSH-monoethyl ester] and/or GST activity (GST-inducing agents, GST inhibitors). Modulation chemotherapy, based on targeting the GSH/GST system, could be directed towards decreasing host toxicity or, alternatively, enhancing drug sensitivity of tumours otherwise resistant to conventional chemotherapy [46]. The most effective approach so far to reducing cellular GSH levels is by inhibition of an enzyme involved in GSH synthesis [γ-glutamylcysteine synthetase (γ-GCS)] with BSO [45]. This inhibitor competes with cysteine in the active site of the enzyme. Numerous reports support a possible role for BSO in increasing the sensitivity of tumour cells to a number of cytotoxic agents, as well as reversing the acquired resistance to these agents [26]. Inhibition of GST activity may lead to decreased drug detoxification and hence increased therapeutic potential of anti-cancer agents. The thiol-loop diuretic ethacrynic acid, and the anti-inflammatory agents, indomethacin and sulphasalazine, have been shown to be direct inhibitors of GSTs, and have also been observed to enhance the cytotoxicity of alkylating agents towards resistant cultured cancer cells.

The effects of these GSH and GST modulators on sensitivities of OC1 and OC2 cells to
alkylating agents, such as 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU), melphalan, chlorambucil and cisplatin, were investigated by measuring alterations in drug cytotoxicity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) cell viability assay. Drug dose–response profiles of the cells treated with or without modulators were constructed. In all drug-modulator profiles, average cell-survival values, estimated at each drug concentration, were expressed as a percentage of a control population incubated with the particular concentration of modulator, only. Some combined studies were also carried out to investigate the combined effect of modulators on drug cytotoxicity. In these studies, % cell viability was estimated as a percentage of control cells incubated with the combination of the modulators.

From these cytotoxicity studies, it was observed that depletion of intracellular GSH levels particularly enhanced the cytotoxicity of alkylating agents on OC1 cells, while, in OC2 cells, the protective effect of GSH on alkylating agent cytotoxicity was less important. This might possibly reflect the difference in intracellular GSH levels in these cell lines. In OC1 cells, the GST inhibitors indomethacin and sulphasalazine enhanced the cytotoxicity of the alkylating agents investigated, while ethacrynic acid had little modulatory effect. These modulators were observed to have some effect against the cytotoxicity of BCNU towards OC2 cells, but had no significant effect on other alkylating agents.

Interestingly, while measured intracellular GSH levels were observed to decrease in both cell lines subsequent to treatment with modulatory concentrations of BSO, significant elevations in GSH levels were observed in both cell lines as a consequence of treatment with increasing concentrations of ethacrynic acid. This might result from stress-response rebound synthesis of the γ-GCS gene. Indeed, ethacrynic acid has been shown to induce a number of thiol-dependent and detoxification gene products within human cells, including γ-GCS, GST Pi and MDR-associated protein [47,48]. In addition, it has been postulated that transient reduction both in GSH levels and in GST Pi activity by ethacrynic acid might subject the cell to oxidative stress and protein-thiol modification and, therefore, potentially serves as an induction signal for regulation of phase II detoxification genes [47].

In the OC1 cell line, modulator studies with both ethacrynic acid and BSO resulted in synergistic enhancement of the cytotoxicity of certain alkylating agents. As ethacrynic acid can also consume cellular GSH via GST-catalysed reactions, the observed synergistic effect between ethacrynic acid and BSO on drug cytotoxicity might be attributable to further depletion of GSH when given to BSO-pretreated cells, where GSH resynthesis is blocked despite the stimulatory effect of ethacrynic acid on cellular GSH levels.

Conclusions
Oesophageal carcinoma is one of the most malignant tumours in man. In the present study, intrinsic resistance to alkylating agents was observed in two squamous oesophageal cell lines. The involvement of the GST/GSH detoxification system as a mechanism of alkylating agent resistance in these cell lines was therefore investigated. Chemical modulation of chemotherapy targeted the GSH/GST system using GST inhibitors and the GSH depleting agent, BSO. In the oesophageal cell line expressing higher levels of GST and intracellular GSH, a link between alkylating agent resistance and GST/GSH detoxification was observed. However, in OC2 cells expressing lower GST and GSH, this appeared to be less important, suggesting the involvement of alternative mechanisms of resistance in this cell line. Treatment with ethacrynic acid resulted in an increase in intracellular GSH.

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References

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