Glutathione-conjugate transport by human colon adenocarcinoma cells (Caco-2 cells)

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The secretion of a glutathione-S-conjugate, dinitrophenyl-glutathione (GS-DNP) was studied in the Caco-2 cell, a cultured human colonic adenocarcinoma cell line with many of the characteristics of enterocytes. The labelled glutathione conjugate was generated within the cell by incubation with ^{14}C -labelled 1-chloro-2,4-dinitrobenzene (CDNB). This compound is hydrophobic and enters the cell by simple diffusion. Cells incubated with CDNB at 10 °C form only one metabolite, GS-DNP. After secretion into the medium GS-DNP is partly converted into one or two slightly more hydrophobic products. This must represent hydrolysis of the glutathione moiety by the action of γ -glutamyl-transferase (EC 2.3.2.2.; γ -GT) because the reaction was completely inhibited by acivicin, an inhibitor of γ -GT. Secretion of GS-DNP was a temperature-sensitive, saturable process with an

apparent K_m of 1.03 ± 0.26 nmol/mg of protein and a $V_{\max}$ of 111 ± 17 pmol/min per mg of protein. The secretion was not sensitive to trans-stimulation by extracellular concentrations of GS-DNP up to 2.5 mM. Furthermore the initial GS-DNP secretion rate was sensitive to dissipation of the membrane potential and correlated closely with the cellular ATP content. Caco-2 cells cultured on nitrocellulose filters secreted GS-DNP significantly faster over the basolateral membrane than over the apical membrane (146 ± 25 versus 90 ± 18 pmol/min per mg respectively). Secretion over both membrane domains of the cell was sensitive to ATP depletion. In conclusion, Caco-2 cells contain an active-transport system that is primarily involved in the secretion of glutathione conjugates and that is present in both plasma membrane domains of the cell.

INTRODUCTION

Intestinal epithelium is equipped with a number of enzyme systems, like glucuronyltransferases and glutathione S-transferases (GSTs), which are involved in detoxification of xenobiotics [1]. This is not surprising in view of the fact that the intestinal mucosa should protect the body against toxic and carcinogenic compounds which are ingested via the gastrointestinal tract. Indeed it has been shown in a number of studies that metabolites of compounds like 1-naphthol, paracetamol and ethinyloestradiol are generated within the intestinal epithelium and subsequently secreted into the lumen of the gut, the vascular side, or both [2–5]. Little is known, however, about the molecular mechanism by which these cells secrete the products of such reactions. In order to delineate one of the possible transport systems involved in these secretory processes we have set up a model system *in vitro* with cultured intestinal epithelial cells in which a glutathione-S-conjugate is generated intracellularly. For this purpose we have used the Caco-2 cell line, which is one of several human colon adenocarcinoma cell lines that have been reported to undergo enterocytic differentiation upon culture *in vitro* [6,7]. The advantage is that the Caco-2 cell is a very well-characterized line with many characteristics of normal enterocytes and that it forms polarized monolayers when cultured on plastic dishes or on permeable filters [8].

As a model substrate for transport we have chosen dinitrophenyl-glutathione (GS-DNP). In order to generate this compound within intact Caco-2 cells they are incubated with 1-chloro-2,4-dinitrobenzene (CDNB). The advantages of using this compound are multiple: first it is a hydrophobic compound that easily permeates into cells by simple diffusion; secondly it is an extremely good substrate for GSTs [9]. Peters and co-workers

[10,11] have shown that human intestinal epithelial cells, as well as confluent Caco-2 cells, contain a high level of GST activity. An additional and important advantage of this compound is that only one metabolite, the glutathione-S-conjugate is formed within the cell. In a number of studies it has been shown that GS-DNP is secreted in the liver by an ATP-dependent transporter which is present exclusively in the canalicular (apical) membrane of the hepatocyte [12–14]. In the present paper we have investigated the characteristics and sidedness of the transport system responsible for the secretion of intracellularly generated GS-DNP in Caco-2 cells.

MATERIALS AND METHODS

Chemicals

^{14}C -labelled 1-chloro-2,4-dinitrobenzene (CDNB; 5.79 mCi/mmol) was from Amersham International, Amersham, Bucks., U.K. and [^{14}C]methoxyinulin (5 mCi/g) from NEN, Dreieich, Germany. Acivicin and collagen type I from rat tail were from Sigma, St. Louis, MO, U.S.A. Dulbecco's modified Eagle's medium (DMEM) was obtained from Imperial Laboratories (Andover, Hants, U.K.). Culture plates (24 well) were from Costar, and nitrocellulose filter inserts were from Millipore (type HA, 10 mm diam. and 0.45 μm pore size). All chemicals were of analytical grade.

Cell culture

Caco-2 cells were cultured with DMEM supplemented with 10% (v/v) fetal calf serum, 250 units/ml penicillin and 250 $\mu\text{g}/\text{ml}$ streptomycin. The cells were split every 7 days at a ratio of 1:5. Confluency was reached within 5 days. During experiments the

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; GS-DNP, dinitrophenyl glutathione; HBSS, Hanks balanced salt solution; GST, glutathione S-transferase; γ -GT, γ -glutamyltransferase; DMEM, Dulbecco's modified Eagle's medium; cMOAT, canalicular multispecific-organic-anion transporter; PCA, perchloric acid; TR[−] rats, mutant rat strain with a defect in canalicular secretion of organic anions.

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cells were seeded on collagen type 1 (rat tail)-coated culture dishes or on nitrocellulose membrane filters until 10 days after confluence was reached. Analysis by electron microscopy of fixed filter-grown cells revealed polarization into apical and basolateral domains.

Measurement of GS-DNP secretion

Before the experiment cells were washed with Hanks balanced salt solution (HBSS; 5.8 mM K^+ , 143 mM Na^+ , 1.3 mM Ca^{2+} , 0.8 mM Mg^{2+} , 146 mM Cl^- , 0.8 mM SO_4^{2-} , 0.8 mM P_i , 4.2 mM HCO_3^- , 5.6 mM glucose and 10 mM Hepes, pH 7.4). In transport experiments the cells were loaded with radioactive GS-DNP by incubation with [^{14}C]CDNB. [^{14}C]CDNB was dissolved in dimethyl sulphoxide and diluted 4000-fold in HBSS before addition to the cells at a final concentration of 2 μ M (0.5 ml; 0.9 nmol/mg). The cells were incubated with the radioactive CDNB for 45 min (or as indicated) at 10 °C. After this incubation period a sample was taken from the medium and counted to determine the amount of radioactivity taken up. Then the cells were washed with HBSS at 10 °C; HBSS at 37 °C was added and efflux was measured during incubation at 37 °C by taking a 100 μ l sample from different wells at the indicated time points. In the case of cells grown on filters the [^{14}C]CDNB was divided over both apical and basolateral incubation compartments, or added to the apical compartment alone. The filters were incubated in wells of a 24-well plate with 300 μ l and 200 μ l of HBSS in the basolateral and apical compartments respectively at 10 °C. After incubation with [^{14}C]CDNB samples were taken from the medium of both compartments to measure the amount of radioactivity taken up. Then the filters were washed in a bath with HBSS at 10 °C. Subsequently the filters were placed in a new 24-well plate with HBSS at 37 °C, again with 300 μ l in the basolateral and 200 μ l in the apical compartments. After the indicated time a 100 μ l sample was taken to determine radioactivity by liquid-scintillation counting. Treatment of the cells with acivicin (0.5 mM) was carried out by pre-incubation for 1 h at 37 °C, treatment with deoxyglucose or with high- K^+ medium, took place by pre-incubation for 20 min at 37 °C. High- K^+ medium consisted of HBSS (see above) in which all Na^+ was replaced by K^+ .

H.p.l.c. analysis of CDNB metabolites

Intra- and extra-cellular metabolites of [^{14}C]CDNB were analysed by h.p.l.c. as follows. After incubation with labelled CDNB for the indicated time medium was taken from the cells, the cells were washed with HBSS and extracted by addition of 10% (v/v) perchloric acid (PCA). After centrifugation the supernatant was neutralized by the addition of 3 M KOH/0.5 M Mops and centrifuged again. Medium that was taken from the cells was adjusted to 10% (v/v) PCA by the addition of 70% (w/v) PCA and further treated like the cell extract. The deproteinized and neutralized samples were applied to a C_{18} column (Chromspher, Chrompack, Middelburg, The Netherlands) on an h.p.l.c. system (Waters, Milford, MA, U.S.A.) that was eluted with a gradient from 40% to 80% (v/v) methanol in 0.01 M sodium phosphate buffer, pH 6.0, and a flow rate of 1 ml/min. Radioactivity in the eluate was monitored by a Ramona on-line scintillation counter (Raytest GmbH, Wildbad, Germany). A radioactive standard of GS-DNP was prepared by incubating [^{14}C]CDNB with excess GSH in 10% (w/v) NH_4Cl at 37 °C. The reaction was followed by recording the absorbance at 335 nm. After completion of the reaction the solution was freeze-dried and applied to a silica gel t.l.c. plate that was chromatographed with n-propanol/ H_2O 7:3.

The yellow spot was scraped from the plate and extracted with water.

RESULTS

In order to load cells with GS-DNP we used an incubation protocol in which CDNB was added to the medium at 10 °C to prevent secretion of the formed GS-conjugate. It has been shown that in hepatocytes, which also have high GST activity, the conjugation reaction readily takes place at 10 °C, whereas subsequent secretion is completely inhibited at this temperature.

Figure 1 shows the results of a representative experiment in which we incubated 10-day-old confluent Caco-2 cells in 2 cm^2 culture dishes with 0.5 ml of DMEM containing 1 nmol of [^{14}C]CDNB and followed the uptake of radioactivity with time. Within 20 min of incubation at 10 °C about 80% of the radioactivity present in the medium was taken up by the cells. The cells were subsequently washed to remove residual extracellular radioactivity and then warmed to 37 °C. Upon warming the cells start to secrete intracellular radioactivity. This secretion was virtually absent when the cells were kept at 10 °C. In all subsequent experiments loading of the cells at 10 °C was carried out for 45 min to ensure optimal uptake and conjugation of the label. The metabolites of CDNB were analysed by h.p.l.c. Figure 2 shows h.p.l.c. analysis of a cell extract after 45 min of incubation with CDNB at 10 °C. All intracellular radioactivity co-eluted with a GS-DNP standard, indicating that at 10 °C any CDNB that enters the cell is completely conjugated with glutathione. This observation is in line with the fact that the cells contain excess GSH (4.2 ± 0.2 nmol/mg of protein) compared with the amount of CDNB taken up (0.9 nmol/mg of protein). Similar to the experiment shown in Figure 1 the cells were washed to remove any residual extracellular label, warmed to 37 °C and allowed to secrete radioactivity for 10 min. The radioactivity that was present in the medium was also applied to the h.p.l.c. column. Figure 2 shows that this material also largely co-eluted with the GS-DNP standard, but this peak has one or two shoulders that were slightly retarded on the column compared with GS-DNP. Since these compounds were not present in the intracellular material it was hypothesized that the extracellular action of γ -glutamyltransferase (γ -GT) and dipeptidylpeptidase may give rise to these compounds by hydrolysing the glutathione moiety. In order to investigate this possibility we repeated the experiment in the presence of 0.5 mM acivicin, an inhibitor of

Figure 1 Uptake and secretion of [^{14}C]CDNB by Caco-2 cells

Ten-day-old confluent Caco-2 cells were incubated with [^{14}C]CDNB (1 nmol) at 10 °C for the indicated time and samples were taken to measure the radioactivity in the medium (○). The cells were washed with cold HBSS and subsequently warmed by the addition of HBSS at 37 °C (●) or HBSS at 10 °C (▼) and the cells were kept at that temperature. Again at the indicated time points radioactivity was determined in the medium.

Figure 2 H.p.l.c. analysis of [^{14}C]CDNB metabolites from Caco-2 cells

Caco-2 cells were incubated with [^{14}C]CDNB for 45 min at 10 °C. The cells were washed and either extracted as described in the Materials and methods section or warmed to 37 °C by the addition of warm HBSS. After 10 min the medium was removed from the cells. In a parallel experiment the cells were treated identically after pre-incubation with acivicin (0.5 mM) for 60 min. The samples were analysed by h.p.l.c. as described in the Materials and methods section. The upper trace of the figure shows the elution profile of a mixture of [^{14}C]CDNB and [^{14}C]GS-DNP.

γ -GT. H.p.l.c. analysis confirmed the hypothesis: after inhibition of γ GT by acivicin all medium radioactivity co-eluted with the GS-DNP standard. It was subsequently investigated whether addition of acivicin had any effect on the secretion rate of GS-DNP. Figure 3 shows that this was not the case: cells that were pre-incubated secreted the radioactivity at the same rate as untreated cells. From this result it can be concluded that extracellular metabolism of the GS-conjugate does not influence secretion.

In order to verify that secretion is a carrier-mediated process, the saturability of transport was determined. Ten-day-old confluent Caco-2 cells were loaded as described above with different amounts of GS-DNP ranging from 0.13 to 0.9 nmol/mg of protein and the initial secretion was measured in the first 8 min after washing and warming the cells. All concentrations used were lower than the concentration in the experiments shown in Figures 1–3 so that complete conjugation of CDNB is assured. The results of a representative experiment are depicted in Figure 4 as a Lineweaver–Burk plot. Analysis of the results reveals saturability with an apparent K_m of 1.03 ± 0.26 nmol/mg of protein and a V_{max} of 111 ± 17 pmol/min per mg of protein ($n = 3$).

In a trans-stimulation experiment the nature of the carrier involved in GS-DNP secretion was explored. Cells were loaded

Figure 3 Effect of acivicin on secretion of radioactivity by Caco-2 cells after loading with [^{14}C]GS-DNP

Caco-2 cells were pre-incubated in the presence (○) or absence (●) of 0.5 mM acivicin and subsequently loaded with [^{14}C]GS-DNP by incubation of the cells with [^{14}C]CDNB for 45 min at 10 °C (again in the presence or absence of acivicin). The cells were washed and efflux of radioactivity was measured at 37 °C in the presence or absence of acivicin. Data in the figure represent means $\pm$ S.D. from three separate determinations.

Figure 4 Double-reciprocal plot of the initial GS-DNP secretion rate as a function of the intracellular GS-DNP content

Cells were loaded with labelled GS-DNP as described in Figure 3. The intracellular GS-DNP content was determined by subtracting the amount of label present in the medium after the loading procedure from the added amount. The cells were warmed to 37 °C and the initial secretion rate of GS-DNP was measured. The results are from a representative experiment from a set of three.

with the standard amount of GS-DNP and washed at 10 °C. Then the cells were warmed by addition of warm medium containing different amounts of unlabelled GS-DNP up to 2.5 mM. Figure 5 shows that the addition of unlabelled GS-DNP neither inhibited nor stimulated the initial secretion of labelled GS-DNP. The absence of trans-stimulation of GS-DNP secretion suggests that the carrier involved is highly asymmetric.

The asymmetric behaviour of GS-DNP secretion suggests that it may involve either primary or secondary active transport. In order to investigate this aspect we designed experiments in which membrane potential or cellular ATP levels were reduced. Cells were incubated either in normal HBSS or HBSS in which all Na^+ was replaced by K^+ to dissipate the Na^+ -gradient, and as a consequence the membrane potential. In these media the cells were loaded with GS-DNP and the initial GS-DNP efflux was determined. Replacement of Na^+ by K^+ led to a $35 \pm 10\%$ decrease in the efflux rate. The efficiency of loading with GS-DNP

Figure 5 Unlabelled GS-DNP in the medium of Caco-2 cells does not trans-stimulate [14 C]GS-DNP secretion

Cells were loaded with [14 C]GS-DNP at 10 °C as described in the legends of the previous figures. After washing the cells, HBSS at 37 °C was added containing the indicated amount of unlabelled GS-DNP and the efflux of radioactivity was measured. Results in the figure are expressed as the mean $\pm$ S.D. from three separate measurements.

Figure 6 Effect of ATP depletion on the initial GS-DNP secretion rate from Caco-2 cells

Caco-2 cells were incubated with glucose-free HBSS containing the indicated concentration of deoxyglucose for 20 min at 37 °C. Then the cells were loaded with labelled GS-DNP at 10 °C as in the previous figures. Subsequently the cells were warmed to 37 °C and GS-DNP efflux was measured (a). In a parallel experiment cells were treated identically but the labelled CDNB was omitted. Cells were extracted and the content of ATP (○) and GSH (●) was measured in the extracts (b). Results in the figure are expressed as means $\pm$ S.D. from three separate experiments (* P < 0.05 as compared with control values in the absence of deoxyglucose).

was the same in high- Na^+ and high- K^+ medium. Exchange of K^+ for Na^+ had no effect on the cellular ATP levels or the GSH content within the experimental time frame.

The effect of ATP depletion was determined by incubation of the cells with different concentrations of deoxyglucose in the

Figure 7 Secretion of GS-DNP by Caco-2 cells grown on nitrocellulose filters

Caco-2 cells were grown on nitrocellulose filters until 10 days after confluency. The cells were pre-incubated in glucose-free HBSS in the absence (●) or presence (○) of 0.8 mM deoxyglucose and subsequently loaded with [14 C]GS-DNP by the addition of labelled CDNB at 10 °C to both the apical and the basolateral compartment. The cells were then washed and warmed to 37 °C and efflux of radioactivity into apical (a) and basolateral (b) compartments was measured. Results in the figure are expressed as means $\pm$ S.D. from three separate experiments.

absence of glucose. As Figure 6 shows the cellular ATP level could be titrated in this way with a maximal decrease of 62 % in the presence of 0.8 mM deoxyglucose. Concomitant with the decrease in the ATP level there was a decrease in the initial GS-DNP efflux with maximal inhibition of 51 % as compared with control cells. Figure 6 also shows that reduction of the ATP level had no influence on the GSH content of the cells.

Caco-2 cells form polarized monolayers in culture; in the previous experiments cells were cultured on plastic dishes and therefore the observed secretion mainly involves apical transport. When grown on permeable filters the cells also polarize with the basal membrane on the filter and transport over both apical and basolateral membranes can be measured. In order to assess the relative contribution of apical and basolateral membranes to the secretion of GS-DNP we have grown the cells on filters and measured secretion at both poles of the cells. Cells were grown until 10 days after confluency and integrity of the monolayers was assessed by measuring the transepithelial electrical resistance over the filters, which amounted to $278 \pm 68 \Omega \cdot \text{cm}^2$, which is in line with previously reported resistance for confluent Caco-2 monolayers [8]. Furthermore analysis by electron microscopy of fixed filter cultures revealed the polarized character of the cells in accordance with previous reports. Transepithelial permeability was also measured by addition of [14 C]methoxyinulin to the apical or basolateral compartment of the filters. Inulin transport across these Caco-2 cell layers was < 2 %/hour from either compartment.

Cells were loaded with GS-DNP from both the apical and basolateral side of the cells in an identical manner as in previous experiments. Figure 7 shows that when the cells are washed and warmed to 37 °C radioactivity was secreted from both sides of the cell. The initial secretion rate at the basolateral membrane was significantly higher than over the apical membrane (146 ± 25 and 90 ± 18 pmol/min per mg respectively). Extrapolation of the secreted radioactivity in time revealed a 1 min lag in the basolateral, but not in the apical secretion. This lag may represent the time needed to pass the filter. The relative contribution of the apical and basolateral membrane to GS-DNP secretion did not differ when the precursor molecule CDNB was either applied on both sides of the cell or on the apical side alone (results not shown).

Pretreatment of cells grown on filters with 0.8 mM deoxy-glucose led to the same ATP depletion as in the experiments of Figure 6. Both apical and basolateral GS-DNP secretion were largely inhibited by this ATP depletion; however, inhibition of apical secretion (82 %) was somewhat stronger than the inhibition of basolateral secretion (69 %).

DISCUSSION

Although the liver is viewed as the most important organ involved in detoxification reactions it is generally recognized that intestinal epithelium contains the enzymic machinery to constitute a primary detoxifying barrier against noxious compounds that are present in the gastrointestinal tract [1]. Intestinal epithelium as well as Caco-2 cells contain a high level of GST activity [10,11]. Furthermore the intracellular GSH content of Caco-2 cells (4.2 nmol/mg of protein) is high enough to support rapid intracellular conjugation of substrates like CDNB. Although the Caco-2 cell has become an attractive model system for studies into enterocytic transport of ions [15], amino-acid residues [16,17] and other ligands [18] little is known about the mechanism of secretion of intracellularly formed products of phase I and phase II reactions. In the present paper we demonstrate that GS-DNP generated within the cell is secreted via a temperature-sensitive, saturable transport system. A $V_{\max}$ of 111 ± 17 pmol/min per mg of protein was observed with an apparent K_m of 1.03 ± 0.26 nmol/mg of protein. It should be emphasized that this apparent K_m gives little information about the affinity of the free substrate for the transporter; most of the intracellular GS-DNP will be bound to cytosolic GST.

Our experiments suggest that the GS-DNP transport system has a highly asymmetric character: extracellular concentrations of up to 2.5 mM unlabelled GS-DNP did not stimulate the secretion of radioactive GS-DNP from the cells into the medium. These observations suggest that the transporter is primarily, if not exclusively, involved in secretion of GS-conjugate and not in uptake. In line with this observation are the experiments which suggest that transport is driven by a primary or secondary active mechanism: a strong correlation could be demonstrated between cellular ATP levels and the initial GS-DNP secretion rate. It is excluded that this effect is secondary, since the cellular GSH content remains constant under these conditions and GST activity is an ATP-independent process. In cells grown on filters the apical efflux rate (90 pmol/min per mg of protein) was higher than in cells grown on plastic (60 pmol/min per mg of protein); furthermore the relative inhibition of transport by ATP depletion was stronger in cells grown on filters than in cells grown on plastic. This may indicate that Caco-2 cells grown on filters have a higher expression level of this ATP-dependent transport system than cells grown on plastic. In this context it is interesting to note that in rat hepatocytes a transport system has been described which is responsible for the apical (canalicular) secretion of GS-DNP and a variety of other organic anions including glucuronide-, glutathione-, sulphate-conjugates and GSSG [19,20]. In a system with cultured hepatocytes Lindwall and Boyer [21] have studied GS-DNP secretion and observed similar asymmetric behaviour to that we report here for Caco-2 cells; in these experiments, however, the driving force of the transport mechanism was not investigated. In subsequent studies using isolated canalicular plasma membrane vesicles several groups have demonstrated that this transport system is a directly ATP-dependent organic-anion pump [14,22,23]. In view of its characteristics this transport system has been termed canalicular multispecific-organic-anion transporter (cMOAT) [12]. ATP-

dependent transport systems with very similar characteristics have been characterized in erythrocytes and heart sarcolemma [24,25]. In the hepatocyte, however, this organic-anion transporter is exclusively localized in the canalicular domain of the cell [20]. The results from our present study suggest that in Caco-2 cells a glutathione-conjugate transport system is present in both apical and basolateral membrane domains and that the transport activities in both domains are sensitive to ATP depletion. These results render it likely that the transporter in Caco-2 cells is different from that in the hepatocanalicular membrane. The latter conclusion is in line with observations by de Vries et al. [26] on the intestinal secretion of naphthyl-glucuronide in normal and TR^- rats. The TR^- rat has a hereditary defect in the above described cMOAT. Organic anions like GS-DNP and naphthyl-glucuronide are not secreted into the bile of these rats [20,26]. De Vries et al. [26] have administered 1-naphthol to the isolated perfused rat small intestine; they demonstrated that glucuronide of 1-naphthol is secreted at both the luminal and the vascular side. They also found that in the TR^- rat this secretion is unaffected indicating that glucuronide secretion in small intestine and canalicular membrane of the liver indeed involves different organ-specific gene products. On the other hand Heijn et al. [27] have shown that GS-DNP transport in inside-out vesicles from erythrocytes has very similar characteristics and ATP-dependency as the system in the hepatocanalicular membrane. These observations suggest that the transport of organic anions in organs such as liver, intestine, erythrocyte and heart is catalysed by a family of ATP-dependent transporters. The tissue-specific transporters may be similar but not identical.

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