GLUTATHIONE EXPORT DURING APOPTOSIS REQUIRES FUNCTIONAL MULTIDRUG RESISTANCE-ASSOCIATED PROTEINS*
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Running Title: GSH export in apoptosis require functional MRPs

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GSH is released in cells undergoing apoptosis, and the present study indicates that the multidrug resistance-associated proteins (MRPs/ABCC) are responsible for this GSH release. Jurkat cells released approximately 75-80% of their total intracellular GSH during both Fas antibody- and staurosporine-induced apoptosis. In contrast, Raji cells, a lymphocyte cell line that is deficient in phosphatidylserine externalization, did not release GSH during apoptosis, and other apoptotic features appeared more slowly in these cells. Jurkat and Raji cell lines expressed comparable MRP and OATP/SLCO (organic anion transporting polypeptide) mRNA levels, and MRPI protein levels; however, differences existed in MRPI localization and function. In Jurkat cells, MRPI was largely localized to the plasma membrane and these cells exported the MRP substrate calcein. Calcein release was enhanced during apoptosis. In contrast, Raji cells had little MRPI at the plasma membrane and did not export calcein under basal or apoptotic conditions, indicating that these cells lack functional MRPs at the plasma membrane. GSH release in Jurkat cells undergoing apoptosis was inhibited by the organic anion transport inhibitors MK571, sulfinpyrazone, and probenecid, supporting a role for the MRP transporters in this process. Furthermore, when MRPI expression was decreased with RNAi, GSH release was lower under both basal and apoptotic conditions, providing direct evidence that MRPI is involved in GSH export.

Cells stimulated to undergo apoptosis release GSH into the extracellular space, but the precise mechanisms behind the GSH export and its significance to the apoptotic process are not understood (1). Because GSH regulates many cellular functions, depletion of intracellular GSH can disrupt these processes. Although apoptotic GSH release may be a simple consequence of cell death, there is increasing evidence that it is required for either activation of specific apoptotic signaling pathways, or for proper dismantling of cellular components (1, 2). For example, the decrease in GSH may lead to an increase in reactive oxygen species, which could function as second messengers, or may accelerate mitochondrial damage and apoptosis.

Intracellular GSH levels are controlled in two major ways: by regulating its rate of synthesis inside the cell, and its rate of transport out of the cell (3). GSH depletion during apoptosis has been reported to occur concomitantly with an increase in extracellular GSH, indicating that GSH is exported (4-8). GSH release is likely mediated by transport proteins because it is detected before plasma membrane integrity is lost (5-8), and it can be inhibited by specific drugs (5-8). Reducing GSH export during apoptosis slows down the apoptotic process (6-8), and in some cases it has been reported to increase cell survival (5). Although GSH release is important for maintaining both basal and apoptotic intracellular GSH levels, the mechanisms responsible for GSH transport out of the cell are not well characterized (3). A recent study suggests that the organic anion transporting polypeptide (OATP/SLCO) family of proteins is responsible for apoptotic GSH release (9); however, only indirect evidence was provided for this conclusion.

Under basal conditions, GSH is thought to be released from cells largely via the multidrug resistance-associated protein (MRP/ABCC) family of proteins (3), although the OATPs may also contribute (3, 10, 11). Several members of the MRP family have been shown to mediate GSH transport, including MRP1, MRP2, MRP4, MRP5, and CFTR (3). For the OATPs, rat Oatp1 is thought to release GSH through an exchange mechanism with an organic anion (10) and human OATP8 (OATP1B3) has been implicated in the cotransport of GSH and bile acids (11). OATP8 is the only human OATP implicated in GSH transport thus far.
The present findings indicate that the MRPs, and not the OATPs, are responsible for the GSH export that is observed in cells undergoing apoptosis.

MATERIALS AND METHODS

Materials. Fas antibody clone CH-11 was purchased from MBL International Corporation (Woburn, MA) and MK571 was purchased from Biomol Research Labs (Plymouth Meeting, PA). Fluorescent caspase 3 substrate Ac-DEVD-AMC was from Calbiochem (San Diego, CA), Annexin V-APC was from BD Pharmingen (San Jose, CA), calcein-AM and FM^®4-36 was from Molecular Probes (Eugene, OR). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture. Jurkat and Raji cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Both cell lines were cultured in RPMI 1640 with L-glutamine (Mediatech, Herndon, VA) with 10% fetal bovine serum, and 10 µg/mL gentamycin (Gibco, Grand Island, NY) and incubated at 37°C and 5% CO₂ atmosphere. Experiments were run with 4 x 10^6 cells/mL in KH buffer (Krebs-Henseleit Buffer: 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 0.6 mM MgSO₄, 1.25 mM CaCl₂ and 10 mM Hepes/Tris; pH 7.5) containing 0.5 mM acivicin, an inhibitor of γ-glutamyl transpeptidase activity.

GSH release. Jurkat and Raji cells were treated with an apoptotic inducer after a 20 minute preincubation period at 37°C in KH buffer plus 0.5 mM acivicin. KH buffer was collected to analyze extracellular GSH and cell lysate was analyzed for intracellular GSH release.

Phosphatidylserine externalization. After treatment, Jurkat and Raji cells were stained with Annexin V-APC and propidium iodide in KH buffer containing 2.5 mM CaCl₂. Cells were analyzed for propidium iodide exclusion and increases in Annexin V-APC staining using a Becton Dickinson FACSCalibur flow cytometer (Franklin Lakes, NJ) at the University of Rochester Flow Cytometry Core. Data were analyzed using Cell Quest software, gating out propidium iodide positive cells and including Annexin V positive cells for phosphatidylserine externalization.

Plasma membrane integrity. Plasma membrane integrity was measured by propidium iodide exclusion and by lactate dehydrogenase (LDH) release. Propidium iodide exclusion was measured by flow cytometry. LDH release was measured as described by Vassault (14), and the results are expressed as a percentage of total LDH activity (lysed untreated cells).

Caspase 3-like activity. The caspase 3-like activity assay was based on one provided by BD PharMingen. Briefly, cells were placed in cell lysis buffer (10 mM Tris-HCl, 10 mM NaH₂PO₄/NaHPO₄ pH 7.5, 130 mM NaCl, 1% Triton X-100, and 10 mM sodium pyrophosphate) and frozen at -80°C until activity assay. Cell lysates were combined with 1X HEPES buffer (20 mM HEPES pH 7.5, 10% glycerol, and 2mM DTT) and 30 µM Ac-DEVD-AMC. Caspase 3-like activity was measured using a SPECTRAMax Gemini XS spectrofluorometer (Molecular Devices Corporation, Sunnyvale, CA) at 37°C.

DNA fragmentation. Jurkat and Raji cells were incubated for 20 minutes with 10 µg/mL Hoechst 33258 (Hospira, Lake Forest, IL) cytometer and analyzed with EPICS EpicSoftware (Beckman Coulter) at the CytoLogic software (Beckman Coulter) at the University of Rochester Flow Cytometry Core.

Truncated Bid protein expression analysis. Whole cell lysates were prepared as previously described (16, 17). Briefly, cells were resuspended in western blot lysis buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM KCl, 1.5 mM MgCl₂, 2 mM phenylmethylsulfonylfluoride (PMSF), 200 µg/mL EDTA, and protease inhibitor cocktail at 5 x 10⁶ cells/mL at 4°C for 10 minutes and then homogenized. Sucrose was added to a final concentration of 250 mM and lysate was centrifuged at 800 x g at 4°C for 20 minutes. Proteins were detected using 1:1000 primary antibody for Bid (Cell Signaling, Beverly MA) overnight at 4°C and secondary antibody (1:3000) for one hour at room temperature. Images were detected using a Kodak Digital Science Image Station 440 (Eastman Kodak Company, Rochester, NY).

MRP1 protein expression analysis. Membrane enriched fractions were prepared as previously described (16, 17). Briefly, whole cell lysate was prepared as above and was further centrifuged at 100,000 x g at 4°C for 20 minutes. Cell pellets were
resuspended in 10 mM Tris-HCl (pH 7.4), 125 mM sucrose, 200 µg/mL EDTA, 2 mM PMSF and protease inhibitor cocktail. MRPr1 (Alexis Biochemicals, San Diego, CA) primary antibody was diluted to 1:300 and the secondary antibody was 1:15000.

mRNA expression. Total RNA was isolated using the RNeasy Mini Kit and the RNase-Free DNase set (Qiagen, Valencia, CA). Oligonucleotide primers were designed for portions of the MRP and OATP families of genes as well as for β-actin using Primer Express 1.5 (Applied Biosystems, Foster City, CA) and published sequences from GenBank (Table 1). One primer set was designed that identifies both OATP-C and OATP8 because they are very similar in sequence. Reactions were conducted and analyzed on a Roto-Gene 3000 real time light cycler (Corbett Research, Phenix Corporation, Hayward, CA). PCR analysis was performed on 10-100 ng RNA using Quantitect Sybr Green quantitative RT-PCR kit (Qiagen, Valencia, CA).

Immunofluorescence detection of MRP1. Cells were fixed and stained using the Fix and Perm Cell Permeabilization Kit (Caltag Laboratories, Burlingame, CA). After washing with PBS/5 % goat serum, cells were incubated with 1.25 μg/mL MRPr1 (Alexis Biochemicals, San Diego, CA), 1.25 μg/mL rat IgG2a (Zymed Laboratories, San Francisco, CA) in Reagent B of the Fix and Perm Kit, or PBS/5% goat serum for 30 min at room temperature in a humidified chamber. Rat IgG2a and PBS/5 % goat serum were used as negative controls. Cells were then labeled with a 1:1000 dilution of anti rat IgG Alexa Fluor 647 (Molecular Probes, Eugene, OR) in Reagent B for 30 min at room temperature in a humidified chamber. After washing, 10 nM of SYTOX green nucleic acid stain (Molecular Probes, Eugene, OR) was added for 10 min. Cells were fixed in 3.7 % paraformaldehyde and mounted with Fluoromount G (Southern Biotech, Birmingham, AL).

Confocal laser scanning microscopy. Images were taken with a Leica TCS SP Spectral Confocal microscope (Leica Microsystems, Exton, PA) incorporating an upright DMRE Leica microscope at the University of Rochester Pathology/Morphology Imaging Core. Images were scanned simultaneously with a 488 nm argon laser and a 633 nm helium-neon laser.

MRP transport activity. Cells were incubated with 1 µM calcein-AM at 37°C for 30 minutes, collected by centrifugation, and resuspended in KH buffer plus 0.5 mM acivicin. Apoptotic inducers were added and cells incubated at 37°C for the indicated times. Samples were analyzed on a SPECRATmax Gemini XS spectrofluorometer (Molecular Devices Corporation, Sunnyvale, CA) at 37°C, excitation 485; emission 530. Cells without calcein were measured to detect background fluorescence. Protein was analyzed using the DC protein assay (Bio-Rad, Hercules, CA). The calcein release data were expressed as average fluorescence/mg protein and then converted to percent of calcein released (supernatant) from total calcein made by cells (supernatant + cell lysate calcein).

RNAi knockdown of MRP1 in Jurkat cells. Double stranded siRNAs specific to MRP1 were designed by Dharmacon Inc. (Lafayette, CO). The targeting sequences were sense: 5'-GAUGACACCUCUCAACAAAUU and antisense: 5'-PUUUGUUGAGGGUCAUCCU (Catalog no. MU-007308-00-0020). siCONTROL non-targeting siRNA (Dharmacon Inc) was used as a negative control. Jurkat cells were transfected as described by Ku et al. (18). Briefly, 2 x 10⁶ cells were resuspended in 400 µL culture media and electroporated with 100 nM siRNA and 10 µg pmaxGFP (Amaza Inc, Gaithersburg, MD) at 250 V and 950 µF using the GenePulsar Xcell Electroporation System (Bio-Rad, Hercules, CA). After electroporation, cells were incubated for 24 hours in 200 mL culture media. As controls, cells were electroporated without RNA or were not electroporated. After 24 hours, the cells were resuspended in cold KH buffer and sorted for GFP positive cells at the University of Rochester Flow Cytometry Core using a FACS Vantage SE (Becton Dickinson, Franklin Lakes, NJ). GSH release experiments and total intracellular and extracellular GSH levels were assayed as described above.

Statistical analysis. Statistical analysis was performed using Statview 5. Data were analyzed using one-factor ANOVA and Fishers PLSD posthoc or unpaired t tests where appropriate. In all cases p values of less than 0.05 were considered statistically significant.

RESULTS

GSH release is a feature observed in both intrinsic and extrinsic apoptosis. Jurkat cells treated with Fas antibody released a large amount of intracellular GSH into the media (Figure 1A), confirming the findings of van den Dobblesteen et al. (8). There was a concurrent decline of intracellular GSH (data not shown). Apoptosis was indicated by an increase in phosphatidyserine externalization (Figure 1C), caspase 3 activity (Figure 1E), DNA fragmentation...
Another lymphocyte cell line, the Raji cells, was tested for GSH release during apoptosis in order to examine if this transport process is associated with phosphatidylserine externalization, as Raji cells are deficient in this process (19-22). Raji cells did not externalize phosphatidylserine after Fas antibody exposure (Figure 1D), confirming previous findings (19); however, they exhibited features of apoptosis as indicated by increases in caspase 3 activity (Figure 1F) and DNA fragmentation (Figure 1H), and the presence of tBid (Figure 1J). The increase in apoptotic markers was higher in Jurkat cells and caspase activity appeared more quickly in the Jurkat cells than in the Raji cells (Figure 1). Both the Raji and Jurkat cells had approximately 40 ng intracellular GSH/mg protein; however, GSH was not released in Raji cells treated with Fas antibody (Figure 1B). In addition, GSH was not released even after 6 hours of treatment or when 500 ng/mL Fas antibody was used (data not shown).

Inducing apoptosis chemically with staurosporine also resulted in GSH release in Jurkat cells, but not in the Raji cells (Figure 2). Raji cells also failed to externalize phosphatidylserine after treatment with staurosporine (Figure 2D). Staurosporine elicited other features of apoptosis in the Raji cells, including enhanced caspase activity (Figure 2F) and DNA fragmentation (Figure 2H), and truncation of Bid (Figure 2J). Similarly to Fas antibody treatment, staurosporine led to a higher apoptotic marker expression level in the Jurkat cells when compared with the Raji cells (Figure 2).

**Jurkat and Raji cells express comparable levels of MRPs and OATPs.** MRPs and OATPs are efflux transporters that are highly conserved in evolution. The MRPs and OATPs are highly conserved in evolution and are involved in the transport of a wide range of molecules, including GSH. In Jurkat and Raji cells, the MRPs and OATPs are expressed at comparable levels, and the expression levels are similar in both cell lines. This suggests that the MRPs and OATPs are involved in the transport of GSH in both cells.

**MRP1 is not localized to the plasma membrane and MRP-associated transport activity is not observed in Raji cells.** MRPs and OATPs are generally located either at the plasma membrane or in intracellular vesicles (23-25). These vesicles may regulate transport activity through trafficking to and from the plasma membrane (23). To examine MRP1 subcellular localization in Jurkat and Raji cells, these cells were stained with MRP1 antibody and visualized using confocal laser scanning microscopy. In the Jurkat cells, plasma membrane MRP1 staining was observed (Figure 4B, 4C and 4D), whereas MRP1 staining was largely intracellular in the Raji cells (Figure 4F, 4G and 4H). Note that both Jurkat and Raji cells have large nuclei and very little cytoplasm (Figure 4A and 4E). FM^+1-43FX, a marker for the plasma membrane, confirmed that the MRP1 staining was on the plasma membrane in Jurkat cells and that MRP1 was not on the plasma membrane in Raji cells (data not shown).

To examine whether differences in MRP transport activity between the Jurkat and Raji cells may explain the lack of GSH export in the Raji cells, the export of the MRP substrate calcein was measured in both cell lines. Calcein-AM freely diffuses into cells and is cleaved by non-specific esterases to form the fluorescent product calcein. Calcein is a substrate for MRP transporters, and the overexpression of MRP proteins has been shown to deplete cells of calcein (26, 27). Untreated Jurkat cells released approximately 25% of the calcein over 3 hours (Figures 5A and 5C), suggesting that these cells have functional MRP proteins. Adding Fas antibody (Figure 5A) or staurosporine (Figure 5C) increased the amount of calcein released by Jurkat cells. This increase in calcein release follows a similar time course as the increase in GSH release due to either apoptotic inducer (Figures 1A and 2A). Probenecid inhibited both the basal calcein release and that induced by Fas antibody (Figure 5A) or staurosporine (Figure 5C). MK571 also inhibited both basal and Fas antibody-stimulated calcein export (data not shown).

In contrast to the Jurkat cells, Raji cells loaded with calcein failed to release this compound over 3 hours, and treatment with Fas antibody or staurosporine did not stimulate calcein release (Figures 5B and 5D). Note that both Jurkat and Raji cells had similar levels of total calcein fluorescence (Figures 5E and 5F).
MRP inhibitors decrease GSH export during apoptosis in Jurkat cells. If MRPs are responsible for the apoptotic GSH release in Jurkat cells, then pharmacological inhibitors of MRP transport should reduce this GSH release. Three different chemicals were added to Jurkat cells undergoing apoptosis, MK571, sulfinpyrazone, and probenecid. All three chemicals are commonly used to inhibit MRP transport; however, none are specific inhibitors of the MRPs. Specific MRP inhibitors do not exist. Nevertheless, these chemicals were able to inhibit GSH release stimulated by Fas antibody (Figure 6A-C). Probenecid was the most effective, diminishing Fas-stimulated GSH release to near basal levels (Figure 6C).

The progression of Fas-stimulated apoptosis was also affected by inhibiting GSH release, as measured by phosphatidylserine exposure, caspase activity, and fragmented DNA. All three compounds decreased the appearance of extracellular phosphatidylserine (Figure 6D-F), and caspase 3-like activity (Figure 6G-I). The most striking effect of the MRP inhibitors was observed on DNA fragmentation. The inhibitors prevented any further increase in DNA fragmentation after 3 hours (Figure 6J-L). Additionally, these three compounds decreased GSH release and apoptotic markers during staurosporine-induced apoptosis (data not shown).

The inhibition of GSH release by MK571 and probenecid (Figure 6) differs from Franco and Cidlowski (9), who found a stimulation of GSH release by these compounds, and thus concluded that the OATPs are responsible for the GSH export. To examine whether these differences are due to dose-dependent effects of the inhibitors, Jurkat cells were treated with differing concentrations of MK571 (Figure 7A) or probenecid (Figure 7B), together with Fas antibody to induce apoptosis. None of the concentrations tested stimulated GSH release, and both compounds showed inhibitory effects at the higher doses.

Two additional organic anions, taurocholate (Figure 7C), and estrone 3-sulfate (Figure 7D), were tested to see if they would stimulate GSH release during apoptosis. Taurocholate and estrone 3-sulfate are known substrates of some OATPs. Taurocholate was shown to enhance GSH release by rat Oatp1 (10) and Franco and Cidlowski (9) reported that both taurocholate and estrone 3-sulfate increase GSH release during apoptosis. However, neither of these organic anions increased the amount of basal or apoptotic GSH release (Figures 7C and D), and thus do not support an OATP-like exchange mechanism as the driving force for GSH release during apoptosis. Of significance, instead of stimulating GSH release, estrone 3-sulfate was an effective inhibitor of GSH release (Figure 7D). Because estrone 3-sulfate is also a substrate of MRP1 (28), it may be competitively inhibiting GSH release by MRP1.

RNAi knockdown of MRP1 attenuates GSH release during apoptosis. To directly assess the role of MRP1 in GSH release during apoptosis, Jurkat cells were electroporated with siRNA targeting MRP1 along with a transfection efficiency marker, a green fluorescent protein (GFP) vector. Sorting for GFP fluorescence enriched the population of cells transfected with the siRNA. MRP1 mRNA expression was decreased by about 70 percent in siRNA treated cells (Figure 8A) and MRP1 protein levels were decreased to a similar extent (Figure 8B). Basal GSH release, as well as apoptotic GSH release, was lower when MRP1 expression was decreased (Figure 8C). Control experiments in which cells were transfected with nontargeting siRNA did not result in altered MRP1 expression or apoptotic GSH release (data not shown). These findings provide direct evidence that MRP1 is a major contributor to both basal and apoptotic GSH release.

DISCUSSION

The present findings indicate that the MRP proteins, and in particular MRP1, are involved in the GSH release that is observed in cells undergoing apoptosis, and do not support the recent suggestion that the OATP proteins are mediating this event (9). A role for the MRPs was established using RNAi knockdown of MRP1, pharmacological inhibitors, and by correlating MRP functional activity with GSH export rates in two related lymphocyte cell lines, Jurkat and Raji cells. The present findings also demonstrate that GSH release occurs in both intrinsic (staurosporine) and extrinsic (Fas antibody) apoptosis, and is associated with phosphatidylserine externalization, suggesting this transport event is important for multiple signaling pathways during apoptosis.

Selectively knocking down MRP1 expression using RNAi reduced GSH release during basal
conditions and during apoptosis, providing direct evidence for the involvement of MRP1 in GSH export, and suggesting that MRP1 is the major transporter involved in GSH release in these cells. Because MRP1 is ubiquitously expressed in cells, it could provide a common mechanism for GSH release during apoptosis.

Jurkat cells express several MRP transcripts, including relatively high levels of MRPI, and are able to release calcine, an MRP substrate. Calcine was exported by control Jurkat cells, and this export increased during apoptosis. Both the basal and apoptotic release of calcine were inhibited by probenecid and MK571. Taken together, these findings indicate that MRP transporters are functional in Jurkat cells, and mediate the increased export that is observed in apoptotic cells. In contrast to the Jurkat cells, Raji cells did not export GSH during either intrinsically- or extrinsically-induced apoptosis. The Raji cells had a similar MRP expression profile when compared with the Jurkat cells; however, the Raji cells lacked MRPI at the plasma membrane and were unable to export calcine during basal or apoptotic conditions. These data indicate that MRPI, and perhaps other MRPs, are not properly trafficked to the plasma membrane in the Raji cells, and that this may account for their inability to export GSH or calcine.

In the present study, the organic anion transport inhibitors, MK571, sulfinpyrazone, and probenecid, were able to reduce GSH release and the appearance of apoptotic characteristics during both intrinsically- and extrinsically-induced apoptosis in Jurkat cells. All three inhibitors are known to reduce transport on MRP proteins (29-32), although none are specific for these proteins. Probenecid is thought to affect a wide range of organic anion transporters, including the MRP family (29, 33, 34), the OATP family (35-37), and the organic anion transporter (OAT) family (38-40). Sulfinpyrazone and MK571 appear to be somewhat more selective for the MRPs, and are commonly used to inhibit MRP transporters, although MK571 has recently been reported to inhibit OATP8 (OATP1B3) (41). Jurkat cells do not express detectable levels of OATP8 (9).

In contrast with the present results, a recent study demonstrates that MK571 and probenecid can actually stimulate GSH release (9). However, in this previous study, GSH export was assessed largely indirectly, by measuring the fluorescence produced when cells are treated with monochlorobimane (mBCI) using flow cytometry (9). In this method, mBCI diffuses into cells where it is conjugated with GSH by the glutathione S-transferases, resulting in the formation of the fluorescent bimane-glutathione conjugate. Of significance, the bimane-glutathione conjugate is a substrate for several MRP proteins, notably MRPI, which mediates the export of the bimane-conjugate out of the cells (42, 43). Thus, the intensity of the bimane fluorescent signal is dependent not only on intracellular GSH levels, but also on the amount of mBCI internalized, the activity of the glutathione S-transferases, and the rate of export via the MRP proteins. In addition, as cells fragment into apoptotic bodies, the resulting fragments would have lower fluorescence and may be misinterpreted on flow cytometry as intact cells that have released GSH. In the present study GSH release was measured directly, and MK571 and probenecid did not stimulate GSH release, but rather they inhibited this process.

Although Franco and Cidlowski (9) did attempt to measure GSH more directly in one experiment (Table 2 of their report), the results of this experiment are ambiguous as the recovery of GSH was incomplete. Their data demonstrate that when cells were treated with Fas ligand they lost more than 50% of their GSH, as expected (a decrease from 234 to 110 nmoles GSH/mg protein, or a loss of 124 nmoles GSH/mg protein); however, only a small fraction of this GSH was recovered in the extracellular medium. The amount found in the medium increased from ~5 to 18 nmoles GSH/mg protein, or an increase of only 13 nmoles GSH/mg protein (9). The fate of the remaining 111 nmoles of GSH, or nearly 90% of the GSH lost by the cells, is unknown. Likewise, treatment with MK571 apparently further enhanced GSH loss in cells undergoing Fas ligand-induced apoptosis (the cells lost an additional 32 nmoles GSH/mg protein), yet the amount of GSH in the extracellular medium only increased by 7 nmoles GSH/mg protein (9). The reason for this incomplete recovery is unknown, but is likely an artifact of the analytical method used to measure GSH levels. Because these measurements were made in the presence of acivicin, GSH degradation by the enzyme gamma-glutamyltransferase is unlikely to account for the loss of GSH.

Overall, the present findings indicate that GSH is released on MRPI during apoptosis and that GSH release is playing an important role in the apoptotic process. During conditions in which apoptotic GSH release was inhibited or absent, apoptotic markers increased to a lesser extent. Perhaps GSH is released...
in order to make the apoptotic process more efficient or to activate certain signaling pathways used for apoptosis.

Interestingly, GSH release may also be associated with phosphatidylserine externalization, as both processes are lacking in Raji cells, and both were reduced by GSH transport inhibitors. The mechanism for the outward movement of phosphatidylserine during apoptosis has not been identified, although potential roles for ABCA1 (44), ABCG2 (45), multidrug resistance transporter 1 (MDR1/ABCB1) (46, 47), and MRP1 (48-50) have been suggested. MRP1 is an organic anion pump, and phosphatidylserine, unlike the other major plasma membrane lipids, is negatively charged. Studies have demonstrated that the outward movement of fluorescently labeled phosphatidylserine is reduced in erythrocytes from MRP1 knockout mice (49), and in the presence of compounds known to inhibit MRP1 (50). In addition, Sohnius et al. (48) indicated that the presence of GSH stimulates the translocation of phosphatidylserine analogs on MRP1. However, each of these studies used chemically modified analogs of phosphatidylserine, which may behave differently than native phosphatidylserine, and may thus be recognized as xenobiotics by MRP1 or other MRP proteins.

DNA fragmentation may also be affected by intracellular GSH levels. In the present study, DNA fragmentation reached a plateau when GSH release was decreased with chemical inhibitors. Perhaps the GSH remaining in the cell provides protection for the DNA. Recently, it was reported that the morphology of DNA damage is different in cells that release GSH during apoptosis versus those that do not because preventing GSH release changes the signaling pathway for DNA damage (51). In the present study, the Jurkat cell pathway to DNA damage may have been altered when GSH release was decreased, resulting in a protective effect. Alternatively, the pathway to DNA damage in Raji cells may be unaffected by GSH levels.

In summary, this study is the first to provide direct evidence that GSH is released on MRP1 during apoptosis, and argues against a role for an OATP-like transporter. The data also indicate that GSH release is important for a variety of pathways that are activated in both intrinsically- and extrinsically-induced apoptosis.
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FOOTNOTES

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# These authors contributed equally to this work.

1The abbreviations used are: CFTR Cystic Fibrosis Transductance Regulator; LDH Lactate dehydrogenase; KH Krebs-Henseleit; mBCl Monochlorobimane; MDR Multidrug resistance transporter; MRP Multidrug resistance-associated protein; OAT Organic anion transporter; OATP Organic anion-transporting polypeptide; TBST Tris buffered saline with 0.05% Tween 20

FIGURE LEGENDS

Fig 1. Raji cells do not release GSH or externalize phosphatidylserine during Fas Ab-stimulated apoptosis. Jurkat or Raji cells were untreated (◊) or treated with 250 ng/mL Fas Ab (□). A) Percent of total GSH released from Jurkat cells. B) Percent of total GSH released from Raji cells. C) Percent annexin V positive and propidium iodide negative Jurkat cells. D) Percent annexin V positive and propidium iodide negative Raji cells. E) Activity of caspase 3-like proteases in Jurkat cells. F) Activity of caspase 3-like proteases in Raji cells. G) Flow cytometric analysis of the percentage of Jurkat cells containing SubG1 DNA. H) Flow cytometric analysis of the percentage of Raji cells containing SubG1 DNA. Values are means ± SE, n = 3-5. * Significantly different from control cells, P < 0.05. I) Western blots of control Jurkat cells and cells treated with 250 ng/mL Fas Ab showing Bid and truncated Bid protein expression. J) Western blots of control Raji cells and cells treated with 250 ng/mL Fas Ab showing Bid and truncated Bid protein expression. Western blots are representative of at least 3 independent experiments.

Fig 2. GSH is not released in Raji cells during staurosporine-stimulated apoptosis. Jurkat or Raji cells were untreated (◊) or treated with 2.5 µM staurosporine (□). A) Percent of total GSH released from Jurkat cells. B) Percent of total GSH released from Raji cells. C) Percent annexin V positive and propidium iodide negative Jurkat cells. D) Percent annexin V positive and propidium iodide negative Raji cells. E) Activity of caspase 3-like proteases in Jurkat cells. F) Activity of caspase 3-like proteases in Raji cells. G) Flow cytometric analysis of the percentage of Jurkat cells containing SubG1 DNA. H) Flow cytometric analysis of the percentage of Raji cells containing SubG1 DNA. Values are means ± SE, n = 3-5. * Significantly different from control cells, P < 0.05. I) Western blots of control Jurkat cells and cells treated with 2.5 µM staurosporine showing Bid and truncated Bid protein expression. J) Western blots of control Raji cells and cells treated with 2.5 µM staurosporine showing Bid and truncated Bid protein expression. Western blots are representative of at least 3 independent experiments.

Fig 3. Jurkat and Raji cells express similar mRNA levels for the MRPs and OATPs, and similar levels of MRP1 protein. Real time RT-PCR was performed for the nine MRP genes and CFTR in Jurkat cells (A) and Raji cells (B). Real time RT-PCR was also performed for the OATP genes in Jurkat (C) and Raji cells (D). One primer set that identifies both OATP-C and OATP8 was used. Data are expressed relative to β-actin levels. Values are means ± SE, n = 3-5. E) Western blot for MRP1 protein expression (25 µg of protein was loaded for each sample). The mouse lung sample was included as a positive control and this western blot is a representative example of three experiments.

Fig 4. MRP1 is localized to the plasma membrane in Jurkat cells, but is intracellular in Raji cells. Jurkat (A, B, C and D) and Raji cells (E, F, G and H) were added to slides at a density of 100,000 cells/slide, air dried, fixed and fluorescently labeled with a monoclonal antibody against MRP1 followed by a secondary antibody conjugated to Alexa Fluor 647 (B and F). Nuclei of both cells were labeled with SYTOX green (A and E).
Images were taken using confocal laser scanning microscopy with a 63x oil immersion lens. Panels C and G represent merged images of panels A and B, and panels E and F, respectively. Panels A-C and E-G are 158 μm x 158 μm, panel D is 97 μm x 97 μm and panel E is 85 μm x 85 μm. These are representative pictures of at least 4 independent staining events.

**Fig 5.** Jurkat cells release calcein and this release is stimulated during apoptosis; whereas Raji cells do not release calcein. Jurkat (A, C, and E) or Raji cells (B, D, and F) were untreated (◊) or treated either with an apoptotic stimuli (250 ng/mL Fas Ab or 2.5 μM staurosporine (□), 7 mM probenecid (□)), or a combination of apoptotic inducer and 7 mM probenecid. Panels A-D show the percent of total calcein that is released over 3 hours. Panels E and F represent the total amount of calcein that was present in the cells and in the culture medium during each time point over 3 hours. Values are means ± SE, n = 4. * Significantly different from control cells, P < 0.05.

**Fig. 6.** Compounds that inhibit GSH release also inhibit apoptotic progression. Jurkat cells were either untreated (◊), or exposed to 250 ng/mL Fas Ab (□), an inhibitor (□), or a combination of 250 ng/mL Fas Ab and inhibitor (◊). The three inhibitors used in this experiment were 75 μM MK571 (A, D, G, and J), 5 mM sulfinpyrazone (B, E, H, and K) or 7 mM probenecid (C, F, I, and L). A, B, and C) Percent of total GSH released from cells. D, E, and F) Percent annexin V positive and propidium iodide negative. G,H, and I) Activity of caspase 3-like proteases. J,K, and L) Flow cytometric analysis of the percentage of cells containing SubG1 DNA. Values are means ± SE, n = 3-5. * Significantly different from control cells, P < 0.05.

**Fig. 7.** Organic anions do not stimulate GSH release. Control Jurkat cells (open bars) and Jurkat cells treated with 250 ng/mL Fas Ab (solid bars) were analyzed for GSH release in the presence of organic anions in the culture medium. GSH release due to increasing concentrations of A) probenecid; B) MK571; C) taurocholate; and D) estrone 3-sulfate. Values are means ± SE, n = 3.

**Fig. 8.** Basal and apoptotic GSH release is diminished when MRP1 expression is decreased. Jurkat cells were electroporated with MRP1 siRNA. A) mRNA expression of MRP1 compared with β-actin. B) Western blot for MRP1 and β-actin. Lanes 1 and 4 are control Jurkat cells, lanes 2 and 5 are Jurkat cells electroporated without siRNA, and lanes 3 and 6 are siRNA treated Jurkat cells. C) The ratio of extracellular GSH levels to intracellular GSH levels. Values are means ± SE, n = 3. * Significantly different from control cells, P < 0.05.
| Protein | Gene | GenBank Accession Number | Forward Primer (5’-3’) | Reverse Primer (5’-3’) | Ta (°C) | Amplicon Size (bp) |
|---------|------|--------------------------|------------------------|------------------------|--------|-------------------|
| MRP1    | ABCC1| NM_0004996                | AGGTTGAGCTTCGCTTCTCTG  | GGACTTTGGGGCTCCCTTCTG  | 57     | 174               |
| MRP2    | ABCC2| NM_000392                 | CGCTGCCTGAGGATGACAGAGA| TGGCCGACTCTTAATCTGCC  | 68     | 144               |
| MRP3    | ABCC3| AF085690                 | GGCTCGGGCGAGTACAGCT   | GCCCAAGTAGAAGGAGAGGA  | 61     | 184               |
| MRP4    | ABCC4| AY081219                 | TGGTTGACCTTCGACACTCTG | GGGGAGTCGCTGATAATCTCATCAATG | 62 | 201               |
| MRP5    | ABCC5| NM_005688                | AGGTTGAGCTTGGCTTCACTG | ACCCTCGAGGACTGCTCTG   | 57     | 226               |
| MRP6    | ABCC6| NM_001171                | GCTCTACTCTCCGAAATCAGAG| GCTCTCTGCCATTGATAGCT  | 60     | 226               |
| MRP7    | ABCC10| NM_003450           | GATCTGGGACCTCTGGAGCTG | CGTAAATGCTGATACCTGCA  | 61     | 201               |
| MRP8    | ABCC11| NM_002583              | GCCAAAGTAGAAGGATCGAC  | CGTGAAGATGGTAAAGAAGAG | 58     | 216               |
| MRP9    | ABCC12| NM_001587              | AGCAGAAGAAGCAGGCCAGAG| GCTGTGACAGAAGCAGGTA  | 57.8   | 153               |
| CFTR    | ABCC7| NM_000492              | CTCTACCTGCTTGGAGGCTT  | FGCGCTGAGAGGAGGCTTTC  | 58     | 188               |
| OATP-A  | SLC01A2| NM_0001942         | CTTGGAGCAACAGAAGGCTGAC| TTACAGGATGGAGCAAGAGAGA | 59 | 101               |
| OATP-B  | SLC01B1| NM_0012562            | GCCTTTGGGCTGATGATCTGGA| AGGGTTCTTCTTCTCCAGGAG  | 59     | 144               |
| OATP-C  | SLC01B1| NM_0012564            | GAAGGTTCTTCTTCTTGGATA| CAGAAATCAATGATTAGAAAGGC | 55 | 123               |
| OATP-D  | SLC02A1| NM_0003272            | GCCAGCCTCTGCTTCCAGGAG| GAAGAGGAAAGGATTAGGGCC  | 54     | 110               |
| OATP-E  | SLC02A1| NM_0013543            | GCCAGGCTCTGCTTCTTCTAGG| GCGCTCTGCTGAGCAGTCTGAG | 59     | 104               |
| OATP-F  | SLC02A1| NM_0017435            | GAAGTTCTTCTTCTTCTTCTTGGA| GCTGCCCTTCTTCTTGGC  | 57     | 102               |
| OATP-H  | SLC02A1| NM_1809914            | GAGTTAATGCGTCTCACCTGAGC| CTCACCTCTTTGATAGTGCTG | 59     | 147               |
| OATP-I  | SLC02A1| NM_173482             | GAGGCGAGAGTTTTAACAGC | GAACCTTACAAAGGCTGCTGAG | 55     | 155               |
| OATP-J  | SLC02A1| NM_003581             | TTCTGAGCTTGGAGCTAGAG  | GCTCTCTGCTGCTGCTGAGTTA | 59     | 102               |
| OATP8   | SLC02A1| NM_0018441            | GAAGGTTCTTCTTCTTCTTCTTGGA| CTCCCTTTTGATAGTGCTG | 59     | 147               |
| PGT     | SLC02A1| NM_001841             | CACCTTCTGCTTCTTCTTCTTGGA| CTCCCTTTTGATAGTGCTG | 59     | 147               |

**Table 1.** Primer sequences and annealing temperatures (Ta) for Real Time RT-PCR
Figure 3

A. Jurkat Cells

B. Raji Cells

C.

D.

E. MRP1

~ 190 kD
Figure 4
Figure 5
Figure 6
Figure 7

A. 

B.

C. 

D. 

% GSH release

Probenecid (mM)

MK571 (µM)

Taurocholate (mM)

Estrone Sulfate (mM)

Control

250 ng/ml Fas ab
Figure 8

**Panel A**

- **Y-axis**: MRP1 mRNA expression (relative to β-actin)
- **X-axis**: Control, Electroporated without siRNA, siRNA

**Panel B**

- **Lanes**: 1, 2, 3, 4, 5, 6
- **Proteins**: MRP1, β-actin

**Panel C**

- **Y-axis**: Extracellular/Intracellular GSH
- **X-axis**: Time 0, 90 min Control, 90 min Fas Ab

* Asterisks indicate significant differences.
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