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Glutathione depletion regulates both extrinsic and intrinsic apoptotic signaling cascades independent from multidrug resistance protein 1

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Abstract

Glutathione (GSH) depletion is an important hallmark of apoptosis. We previously demonstrated that GSH depletion, by its efflux, regulates apoptosis by modulation of executioner caspase activity. However, both the molecular identity of the GSH transporter(s) involved and the signaling cascades regulating GSH loss remain obscure. We sought to determine the role of multidrug resistance protein 1 (MRP1) in GSH depletion and its regulatory role on extrinsic and intrinsic pathways of apoptosis. In human lymphoma cells, GSH depletion was stimulated rather than inhibited by pharmacological blockage of MRP1 with MK571. GSH loss was dependent on initiator caspases 8 and 9 activity. Genetic knock-down (>60%) of MRP1 by stable transfection with short-hairpin small interfering RNA significantly reduced MRP1 protein levels, which correlated directly with the loss of MRP1-mediated anion transport. However, GSH depletion and apoptosis induced by both extrinsic and intrinsic pathways were not affected by MRP1 knock-down. Interestingly, stimulation of GSH loss by MK571 also enhanced the initiator phase of apoptosis by stimulating initiator caspase 8 and 9 activity and pro-apoptotic BID cleavage. Our results clearly show that caspase-dependent GSH loss and apoptosis are not mediated by MRP1 proteins and that GSH depletion stimulates the initiation phase of apoptosis in lymphoid cells.

Keywords

Glutathione; extrinsic; intrinsic; MRP1; multidrug-resistance; MK571

INTRODUCTION

Reduced glutathione (GSH) is the most abundant low-molecular-weight thiol in animal cells and is the major determinant in the redox potential of the cell. It is involved in many cellular processes, including antioxidant defense, drug detoxification, signaling, and proliferation [1, 2]. GSH is essential for cell survival as the GSH-depleted knock-out mouse of γ -GCS dies

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from massive apoptotic cell death [3]. Furthermore, intracellular GSH depletion is an early hallmark in the progression of cell death in response to different apoptotic stimuli [4–6]. We and others have shown a correlation between GSH efflux and the progression of apoptosis, while inhibition of GSH loss rescues cells from apoptosis [4, 5, 7]. Glutathione depletion has been shown to directly modulate both the formation of the permeability transition pore and the activation of caspase 3 [8–10]. In addition, *in vitro* studies have demonstrated that a reduction in the intracellular GSH content is necessary for the formation of the apoptosome [11]. Moreover, high intracellular GSH levels have been associated with an apoptotic resistant phenotype [12–14].

GSH depletion during apoptosis induced by diverse apoptotic stimuli, such as death receptor ligands, has been reported to be mediated by the activation of a plasma membrane efflux transport [4, 5, 7, 15–18], and previous studies, have shown that FasL-induced GSH efflux is mediated by a transporter [7, 15, 16]. Inhibition of GSH depletion under these conditions is able to rescue cells from apoptosis [5, 7, 16]. However, conflicting results exist regarding the molecular identity of the transporter involved in GSH depletion. Several studies have suggested a role for multidrug resistance proteins (MRP) in GSH depletion [16, 18–20]. However, we and others have demonstrated that inhibition of MRP-mediated transport accelerates apoptosis and GSH loss [7, 21, 22].

In this work we sought to evaluate the role of MRP1 in GSH depletion and apoptosis, determine the signaling cascades regulating GSH loss and identify its role in regulating both intrinsic and extrinsic signaling cascades. We present both pharmacological and genetic evidence demonstrating that GSH depletion and apoptosis is not mediated by MRP1 proteins. Furthermore, we show a direct role of initiator caspases in GSH loss.

MATERIALS AND METHODS

Reagents

RPMI 1640, penicillin/streptomycin and heat-inactivated Fetal Calf Serum were from GIBCO/Invitrogen Co. (Carlsbad, CA). MK571 was from BioMol (Plymouth, PA). Monochlorobimane (mBCl), 5-carboxyfluorescein diacetate (CFDA), JC-1 and Alexa Fluor 488 anti-rabbit were from Molecular Probes Inc. (Eugene, OR). FITC-conjugated anti-MRP1 monoclonal antibody was from BD Biosciences (San Diego, CA). Antibodies against cleaved caspase 3 (Asp 175), cleaved caspase 8 (Asp 391/18C8), caspase 8, 9, 3, 6, 7, BID, Poly (ADP-ribose) polymerase (PARP), lamin and α -fodrin were from Cell Signaling Technology Inc (Beverly, MA). Caspase inhibitors Z-VAD-FMK (pan-caspase), Z-IETD-FMK (caspase 8), Z-LEHD-FMK (caspase 9), AC-DMQD-CHO (caspase 3) and AC-DNLD-CHO (caspase 3,7) were from Calbiochem (EMD Chemicals USA). All other reagents were from SIGMA/Aldrich (St. Louis, MO).

Cell culture and media

Human leukemia Jurkat cells (E6.1 clone) were obtained from American Tissue Culture Collection (Manassas, VA). Jurkat cells genetically deficient in caspase-8 (C8DF, clone I9.2), FADD (FADDDF, clone I2.1) and the parental wild-type cells (WT, clone A3) were kind gifts from Drs. P. Juo and J. Blenis (Harvard Medical School, Cambridge, MA) [23, 24]. Caspase-9 deficient Jurkat cells (C9DF, JMR clone) and caspase-9 reconstituted JMR cells (C9RE, F9 clone) have been described previously [25]. Cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 4 mM glutamine, 31 mg/l penicillin, and 50 mg/l streptomycin at 37 °C, 7% CO₂ atmosphere. Cells ($5-7 \times 10^5$ cells/ml) were incubated with either Fas ligand (FasL) (Kamiya Biomedical Co. Seattle, WA), cycloheximide, etoposide, staurosporine or treated with ultraviolet C light (UVC light in a

UV Stratalinker 1800, Stratagene La Jolla, CA) for the time indicated for the induction of apoptosis. In media containing high glutathione (+ GSH) or N-acetyl-cysteine (NAC), NaCl was substituted with 25mM GSH or 10mM NAC maintaining the same osmolality of the media. Media osmolality was measured on a Wescor 5500 vapor pressure osmometer (Logan, UT).

Knockdown of MRP1/ABCC1

Knockdown experiments were designed according to previous studies [26]. Cells were stably transduced with mission short hairpin small interference RNA (shRNA) (Sigma-Aldrich). Cells were infected with lentiviral pLKO.1 shRNA vectors from the Sigma Mission shRNA library including TRCN0000059363/NM_004996.2-1128s1c, named **4996-3**, Sequence:

CCGGCCTCTCTGTTTAAGGTGTTATCTCGAGATAACACCTTAAACAGAGAGGTTTT
TG; TRCN0000059364/NM_004996.2-4207s1c1, named **4996-4**, Sequence:

CCGGCCTGGGCTTATTCGGATCAACTCGAGTTGATCCGAAATAAGCCCAGGTTT
TTG; TRCN0000059365/NM_004996.2-1675s1c1, named **4996-5**, Sequence:

CCGGCCACATGAAGAGCAAAGACAACCTCGAGTTGTCTTTGCTCTTCATGTGGTTTT
TG; TRCN0000059366/NM_004996.2-3889s1c1, named **4996-6**, Sequence:

CCGGCCTCTCAGTGTCTTACTCATTCTCGAGAATGAGTAAGACACTGAGAGGTTTT
TG; and TRCN0000059367/NM_004996.2-2552s1c1, named **4996-7**, Sequence:

CCGGGCTGACATTTACCTCTTCGATCTCGAGATCGAAGAGGTAATGTCTAGCTTTT
TG, selected in medium containing 3 µg/ml Puromycin after 48 h post-transfection. As

control we used a non-target shRNA pLKO.1 (SHC002, Sequence:

CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTGTTTT
T) coding for the puromycin resistance gene and containing a sequence that should not

target any known human or mouse gene (scramble), but will engage with the RNA-induced silencing complex (RISC). All lentiviruses were packaged in HEK293T cells according to established protocols [27]. HEK293T cells were transiently transfected with pMD2G, psPAX2, and transfer vector containing the shRNA sequence of interest using Lipofectamine 2000. Supernatant was collected 48 h post-transfection and concentrated by centrifugation at $50,000 \times g$ for 2 h. The pellets were resuspended in PBS and used for infection.

Fluorescence Activated Cell Sorting (FACS)

Apoptotic parameters were analyzed by FACS, using a BD LSR II flow cytometer/BD FACSDiva Software (Becton Dickinson, San Jose, CA) for data analysis. Cells were analyzed at a cell density of $5-7 \times 10^5$ cells/ml, and 1×10^4 cells events were collected. Fluorophores were diluted in dimethyl sulphoxide (DMSO) and preloaded at 37 °C, 7% CO₂. The final concentration of DMSO never exceeded 0.1%. When indicated, propidium iodide (PI) was added to a final concentration of 10 µg/ml to assess the loss of membrane integrity. Sequential analysis of the distinct fluorophores was used and cells with increased PI fluorescence were excluded during the analysis. Cells were analyzed in FL-3 fluorescence for PI (488 nm excitation, 695/40 nm emission). Histograms and plots in all cases are representative of at least 3 different experiments.

Changes in intracellular glutathione content, GSH_i

Cells were preloaded for 10 min with 10 µM mBCl, which forms blue-fluorescent adducts with intracellular glutathione [28]. Immediately prior to flow cytometry examination, PI was added. For mBCl, cells were excited with a Violet (UV) 405 nm laser and emission was acquired with a 440/40 filter. Changes in the GSH_i are reflected by the appearance of

populations of cells with differences in mBCL fluorescence. Populations were gated using contour plots of mBCL-fluorescence versus forward scatter as previously described [7, 21].

FACS immunolabeling assay

Samples were fixed and permeabilized using the Cytotfix/Cytoperm kit (BD) for 30 min at room temperature (RT) according to manufacturer's specifications. Cells were then stained with FITC-conjugated rabbit anti-MRP1 or anti-cleaved caspase 8. For cleaved caspase 8 analysis, samples were subsequently stained with Alexa Fluor 488 anti-rabbit. FITC/Alexa 488 fluorescence was detected using an Argon 488 laser with 530/30 (FL-1).

CFDA retention and 5-carboxyfluorescein (CF) efflux assay, for functional assessment of MRP activity

Cells were incubated with 1 μ M CFDA for 1 h at 37 °C. CFDA diffuses into cells where it is cleaved by intracellular esterases resulting in fluorescent carboxyfluorescein (CF), which is a substrate of ABCC/MRP transporters [29]. Cells were then resuspended in CFDA-free medium and allowed to efflux CFDA for the time indicated at 37 °C. For CF-fluorescence, samples were analyzed for FL-1 fluorescence. MRP1 activity is directly correlated with CF loss and is inversely associated with CFDA retention.

Changes in forward scatter (cell size)

Cell size was determined as changes in the forward scatter light pattern of the cells. Cells were excited with an Argon 488 nm laser. The forward-angle light scatter (FSC) relates to cell diameter.

Analysis of Plasma Membrane Lipid Symmetry

Externalization of phosphatidylserine during apoptosis was determined by its recognition with annexin V conjugated to FITC (Trevigen, Gaithersburg, MD). Samples were incubated with annexin-FITC and PI according to the manufacturer's instructions. Annexin-FITC/PI-stained samples were diluted in annexin binding buffer and examined immediately by FACS. FITC fluorescence was analyzed in FL-1. Early apoptotic cells are defined as having annexin positive, PI negative staining. Late apoptotic and non-viable cells are both annexin and PI positive.

Loss of mitochondrial membrane potential (MMP)

Changes in the mitochondrial membrane potential were measured by flow cytometry using JC-1. Thirty minutes prior to cytometric analysis, JC-1 was added to a final concentration of 10 μ M and incubated at 37 °C, 7% CO₂ atmosphere. Samples were examined on a FL-1 versus FL-2 (Argon 488 laser with 585/42 filter, FL-2) dot plot. JC-1 has dual emission depending on the state of the mitochondrial membrane potential. JC-1 forms aggregates in cells with a high FL-2 fluorescence indicating a normal mitochondrial membrane potential. Loss of the mitochondrial membrane potential results in a reduction in FL-2 fluorescence with a concurrent gain in FL-1 fluorescence as the dye shifts from an aggregate to monomeric state.

Analysis of Caspase 3/7-like activity

Caspase-3/7-like activity was determined using a CaspaTag in situ assay kit (Chemicon, Billerica, MA) according to the manufacturer's instructions, which utilize the carboxyfluorescein (FAM) labeled fluoromethyl ketone (FMK) inhibitor probe (DEVD). These probes are derivatives of benzyloxycarbonyl-peptide (caspase recognition sequence)-FMK caspase inhibitors, enter the cell and irreversibly bind to caspase 3, 7. Briefly, 1 h

before cytometric analysis, cells were stained with CaspaTag reagent working stock. Immediately before cytometric analysis, the cells were washed and resuspended in PBS containing PI. Cells were examined in FL-1 for CaspaTag.

Protein extraction and western immunoblotting

Cells were pelleted, washed once with ice-cold PBS, and lysed in buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton X-100 and protease inhibitors (Complete Mini protease cocktail, Roche, IN). Samples were sonicated, centrifuged, and pellets discarded. Then, samples were assayed in a Beckman DU650 spectrophotometer for protein concentration using Biorad Protein Assay (BIORAD, Hercules, CA) and cell extracts were normalized to equal protein concentration. Loading buffer containing glycerol, sodium dodecyl sulfate (SDS), and bromophenol blue was added, and samples were denatured at 99 °C for 5 min. Protein extracts, 50 µg/sample, were separated by SDS-Polyacrilamide gel electrophoresis (PAGE) on 4–20% gradient polyacrilamide tris/glycine gels (Novex, Invitrogen, CA) and transferred to nitrocellulose. Membranes were blocked in Tris-buffered saline (TBS) containing 0.05% Tween, 10% nonfat dry milk and 2% bovine serum albumin (BSA). Antibodies were diluted in TBS containing 0.05% Tween, 5% nonfat dry milk and 1% BSA. Blots were incubated with the corresponding primary antibody (1:1000) overnight. Then, blots were incubated with the corresponding horseradish peroxidase-linked secondary antibody (Amersham Biosciences, Piscataway Corp., NJ) diluted 1:5000. Blots were then visualized on film with the ECL chemiluminescent system (Amersham Biosciences). Blots were subsequently stripped using 65 mM Tris-HCl pH 6.7, 100 mM β-mercaptoethanol, 2% SDS buffer (56°C, 42 min) and reprobed for α-tubulin to verify equal protein loading.

Statistical analysis

When indicated, significances of differences in mean values were calculated using the two-tailed Student's t-test. The number of experiments performed is indicated in the corresponding legend of the figures. FACS plots and western blot images are representative of at least 3 independent experiments

RESULTS

Initiator caspases regulate GSH depletion

Using flow cytometry to analyze apoptosis at the single cell level, we have previously reported that GSH depletion is required for the activation of executioner caspases and the demise of cells upon death receptor-activation. Furthermore, inhibition of *de novo* GSH synthesis stimulates FasL-induced apoptosis. However the role of initiator caspase activity in GSH loss has not been clearly determined [7]. Figure 1A shows that pan-caspase inhibitor Z-VAD-FMK and initiator caspase 8- and 9-like activity inhibitors Z-IETD-FMK and Z-LEHD-FMK fully prevented GSH loss by activation of the death receptor pathway with FasL in Jurkat cells. Apoptosis is characterized by its stochastic/asynchronized progression that conveys the appearance of distinct populations of cells at different stages of the cell death process [7, 30]. GSH loss is observed by the appearance of a population of cells (*light grey*) with a decreased GSH content. In contrast, GSH loss remained unaffected in the presence of the executioner caspase-like activity inhibitors AC-DMQD-CHO (caspase 3) and AC-DNLD-CHO (Caspase 3/7).

Figures 1B and C shows that FasL-induced GSH loss was completely abolished by either FADD or caspase 8 deficiency demonstrating that formation of the DISC signaling complex and activation of initiator caspases are required for GSH loss. In contrast, the absence of caspase 9 only partially reduced GSH loss and remained sensitive to high doses of FasL

(Figures 1D–E). Furthermore, re-introduction of caspase 9 (C9RE in caspase 9-deficient cells enhanced GSH loss by FasL. These results agree with the role of both 8 and 9 in cell death induced by death receptors in type II cells (Jurkat), and are not ascribed to alterations in any other apoptotic signaling molecule as shown in Figures 1B and D.

Downregulation of MRP proteins and MRP transport activity does not regulate GSH depletion and/or apoptosis

GSH loss induced by FasL is mediated through the activation of an efflux transport mechanism for GSH extrusion rather than due to its oxidation to GSSG or loss of membrane integrity [7, 15, 21]. MRP1 proteins have been proposed to mediate GSH efflux during apoptosis [7, 16, 21, 30, 31]. However, we previously reported that inhibition of MRP1 with a variety of drugs including MK571, stimulates rather than inhibits GSH loss and its accumulation in the extracellular space upon activation of the death-receptor pathway [7]. Figure 1A shows that MK571, at a concentration known to inhibit MRP1 activity [7, 32], stimulated GSH loss in response to FasL. MK571-mediated stimulation of GSH loss was observed as a decrease in the number of cells with high GSH content (*in black*), and the appearance of a second population of GSH-depleted cells (*dark grey*) that we have previously demonstrated is associated to later stages of apoptosis and an increase in cellular fragmentation and plasma membrane blebbing [7, 30]. This was corroborated by the absence of this secondary population in the presence of executioner caspase inhibitors (AC-DMQD-CHO and AC-DNLD-CHO). GSH depletion stimulated by MK571 was completely prevented by inhibition of initiator caspases. These results demonstrate that MK571 stimulates caspase-dependent GSH loss.

To further elucidate the role of MRP1 transporters we used stable knockdown of MRP1 in Jurkat cells transduced with shRNA against MRP1. Jurkat cells express MRP1 proteins as determined by FACS analysis using FITC-conjugated MRP1-antibody (Figure 2A). From five shRNA vectors stably expressed in Jurkat cells, only two sequences, 4996-3 and 4996-4, decreased the expression levels of MRP1. Figure 2B shows that shRNA vectors 4996-3 and 4996-4 significantly decreased MRP1 expression (35% and 70% respectively) while non-target shRNA had no effect on MRP1 levels. Downregulation of MRP1 expression by shRNA vectors correlated with downregulation of its activity as evidenced by an increased CFDA accumulation and decreased CF efflux (Figures 2C and D). Because the selection process of stably transduced cells might alter cell size of Jurkat cells and an increase in CFDA uptake, we determined Jurkat's mean cell volume using forward scatter light pattern. As shown in Figure 2C an increase in CFDA accumulation was not related to alterations in cell size.

We next evaluated the role of MRP1 downregulation in GSH loss and apoptosis. As shown in Figure 3A and C, MRP1 knockdown did not inhibit GSH depletion induced by FasL at any concentration tested. In fact, a significant stimulation of GSH depletion was observed on the shRNA clone (4996-4) which corresponds to the highest downregulation of MRP1. Similarly, GSH loss induced by a variety of cytotoxic drugs that trigger the mitochondrial pathway by either impairment of protein synthesis (cycloheximide), inhibition of topoisomerase II (etoposide), protein kinase inhibition (staurosporine) or stress (UVC radiation), was unaltered by downregulation of MRP1 (Figure 3B and D). Additionally, apoptosis induced by FasL (Figure 4A–C) or the mitochondrial intrinsic pathway (Figure 5) also remained unaffected by knockdown of MRP1. Apoptosis was determined by the externalization of phosphatidylserine (Figure 4A and 5A, *light grey population*) with subsequent loss of plasma membrane integrity (*dark grey population*), activation of executioner caspases 3, 6 and 7 and cleavage of their substrates PARP, α -fodrin and lamin (Figure 4C). Thus, we conclude that a reduction in MRP1 proteins does not alter any of the

biochemical/morphological alterations associated with apoptosis at any concentration of FasL. These results clearly demonstrate that MRP1 transporters neither mediate GSH depletion, nor they regulate apoptosis by either the intrinsic or extrinsic pathways.

Glutathione loss regulates the initiation phase of apoptosis by its feedback loop through the mitochondrial pathway

We previously showed that GSH loss during FasL-induced apoptosis is stimulated by the presence of a wide variety of structurally unrelated agents (taurocholic acid, estrone sulfate, probenecid and MK571), and inhibited by high extracellular GSH concentrations [7, 21]. Furthermore, MK571 is a potent stimulator of FasL-induced GSH efflux in Jurkat cells, and thus GSH loss [7]. We have also demonstrated that GSH efflux is necessary for the progression of the execution phase of apoptosis [7]. However, activation of caspases is not a strictly unidirectional event since amplification of the apoptotic signals are mediated in part through positive feedback loops whereby activated executioner caspases 3, 6, 7 can proteolytically cleave initiator caspases to further enhance their activity. Results presented here show that GSH loss is dependent on initiator caspase activity, but whether there is a reciprocal role of GSH loss in executioner caspase signaling is unknown. We observed that high extracellular GSH, which prevents GSH loss and executioner caspase activation [7] also inhibits initiator caspase 8 and 9 cleavage (Figure 6A and C) as well as cleavage of the pro-apoptotic BCL-2 Interacting Domain (BID) (Figure 6B). Conversely, stimulation of GSH depletion by MK571 enhanced caspase 8 activation as well as BID cleavage observed as both a decrease in its full-length and/or an increase in its cleaved form (Figure 6D–F). Both phenomena relate to each other as the enhanced caspase 8 (Figure 7A and C), 9 (Figure 7E) and BID cleavage (Figure 7D) observed in the presence of MK571 were fully prevented by high GSH concentrations. Finally, loss of mitochondrial membrane potential (MMP) was also shown to be stimulated by MK571 and inhibited by high GSH medium (Figure 7B). These results clearly show that although GSH loss depends on initiator caspase activation, perhaps it stimulates the initiation phase of apoptosis through the mitochondrial feedback loop. This notion was further corroborated by the inhibitory effect of caspase 9 deficiency in both caspase 8 activation and loss of mitochondrial membrane potential (Figure 7F and G).

We have previously reported the requirement of GSH depletion in the execution phase of apoptosis by activation of the extrinsic death receptor pathway [7]. We next proceeded to determine the role of GSH in the intrinsic mitochondrial pathway. Because any effect that MK571 might have on apoptosis induced by cytotoxic drugs could be ascribed to their increased accumulation by inhibition of the MRP transporters, we activated the intrinsic mitochondrial pathway via stress induced by UVC radiation. Figure 8A–B demonstrates that apoptosis, determined by activation/cleavage of executioner caspase activity/cleavage and externalized phosphatidylserine, was reduced by extracellular GSH. MK571 induced a significant increase in the processing of executioner caspases, which was also prevented by high GSH media. Similar to observations with FasL-induced GSH loss, UVC-induced glutathione depletion was prevented by initiator caspase 9 deficiency.

DISCUSSION

Changes in the intracellular milieu during apoptosis (permissive apoptotic environment) are determinants in the activation of the apoptotic machinery [6, 33–35]. GSH loss is a common feature of apoptosis induced by different stimuli, and we and others have clearly demonstrated that GSH depletion in FasL-induced apoptosis is mediated by its efflux rather than its oxidation [5, 7, 15, 16]. We previously reported that GSH depletion is necessary for the activation of execution phase of FasL-induced apoptosis [7]. However, the signaling pathways and mechanisms regulating GSH loss are unknown. In this study, we report for the first time that although GSH depletion depends on the activation of initiator caspases, it also

regulates initiator caspase activation through the mitochondrial feedback loop. Finally, we present pharmacological and genetic evidence that GSH depletion is independent from MRP1 activity.

GSH depletion during apoptosis induced by agents that by themselves induce oxidative stress has been reported to be mediated by GSH oxidation to GSSG by reactive species of both oxygen (ROS) and nitrogen (RNS), or by its conjugation to highly reactive compounds [36–38]. In contrast, apoptosis induced by distinct stimuli, such as activation of death receptors, has been reported to be mediated by the activation of a plasma membrane efflux transport and GSH efflux [4, 5, 7, 15, 16, 18]. Inhibition of GSH depletion under these conditions is able to rescue cells from apoptosis [5, 7, 16]. Several studies have suggested that multidrug resistance proteins (MRP) mediate GSH efflux during apoptosis [16, 18–20]. The MRPs act as transporters of GSH, GSSG, and GSH-adducts and require the hydrolysis of ATP for its transport activity [39]. Pharmacological activation of MRPs induces apoptosis by GSH depletion [20, 40]. Paradoxically, we and others have demonstrated that MRP inhibitors accelerate apoptosis [7, 21, 22, 41]. More specifically, pharmacological inhibition of MRP1 stimulated GSH loss induced by death receptor activation [7]. Some reports have demonstrated that in some cell types, at higher doses, inhibitors of MRP1-mediated drug transport such as MK571 stimulate GSH-efflux via MRP1 [42, 43]. However, we have previously reported that neither low $<50\ \mu\text{M}$, nor high $>50\ \mu\text{M}$ concentrations of MK571 prevent GSH depletion and apoptosis [7], which suggests that stimulation of GSH loss by MK571 is independent of MRP1.

For example, contradictory results were reported by Hammond et al. [16] demonstrating that both inhibition and knockdown of MRP1 resulted in a significant reduction of GSH loss and apoptosis induced by both intrinsic and extrinsic pathways in the same experimental model [16]. Hammond and coworkers [16] reported that MK571 and probenecid significantly inhibit FasL-induced apoptosis. Although the authors showed that siRNA knockdown of MRP1 decreased GSH loss, its effect on apoptosis was not evaluated [16]. Curiously, the same group recently reported that overexpression of MRP1 protects rather than stimulates Fas-induced apoptosis [31]. These contradictory results were explained as an effect of MRP1 overexpression inducing an increase in intracellular GSH levels, no attempt was made to corroborate this by GSH synthesis inhibition.

Organic anion transporting polypeptides (OATP) have also been proposed to mediate GSH efflux by a GSH/OA⁻ exchange. However, recent studies have suggested that GSH/OA⁻ exchange is not mediated by this family of transporters [44, 45]. We have proposed a role for an OATP-like transporter in GSH depletion based on the observation that a variety of structurally unrelated anions, including MK571, stimulate GSH depletion. However, there is a possibility that GSH efflux might be mediated by a different and still uncharacterized entity. Other MRP proteins have also been reported to mediate GSH and GSSG efflux including MRP2, 4 and 5 [46, 47], which are similarly inhibited by MK571 [48, 49]. However, the role of GSH efflux mediated by these other MRPs in apoptosis has not been studied in detail. The cystic fibrosis transmembrane conductance regulator (CFTR) has been suggested to mediate transport of GSH during apoptosis [50]. Connexins and glutamate/aspartate transporters (GLAST) have also been suggested to mediate the efflux of GSH in distinct cell types [51–53]. More recently, another ATP-binding cassette (ABC) transporter, the subfamily G member 2 (ABCG2) was identified in human epithelial cells as a GSH efflux transporter but its role in apoptosis has not been studied [54]. It is clear then that further studies are required to elucidate the molecular identity of the transporter(s) mediating GSH during apoptosis.

The signaling cascades that regulate the progression of apoptosis have been extensively studied and characterized, and both extrinsic and intrinsic pathways have been described for the activation of apoptosis. Induction of apoptosis via extrinsic pathways is triggered by the activation of death receptors such as Fas (CD95/Apo-1) activated by Fas ligand (FasL), which leads to the formation of the death-inducing signaling complex (DISC) through the recruitment of the FADD, caspase 8 and the cellular FLICE-inhibitory protein (FLIP). Initiator caspase 8 further amplifies the apoptotic cascade by activation of executioner caspases (3, 6, and 7). In cells that have lower levels of DISC formation and thus, reduced caspase 8 activation (Type II cells such as Jurkat), the progression of apoptosis relies on an amplification loop induced by caspase 8-dependent cleavage of the Bcl-2-family protein BID, translocation to the mitochondria, and subsequent release of cytochrome c (Cyt C) [55]. The intrinsic mitochondrial pathway of apoptosis is activated by a wide variety of stimuli including cytotoxic agents, stress and cytokine withdrawal. Activation of the mitochondrial pathway mediates the release of Cyt C that is associated with the opening of the mitochondrial outer membrane (MOMP) and loss of the mitochondrial membrane potential (MMP). The release of Cyt C leads to the recruitment of Apaf1 into an apoptosome and activates caspase-9 to further regulate executioner caspases [56].

GSH depletion has been shown to regulate both extrinsic and intrinsic apoptotic signaling cascades at distinct checkpoints. GSH depletion can predispose cells to apoptosis or directly trigger cell death by modulation of both the permeability transition pore formation and the activation of executioner caspases [8–10, 57, 58]. *In vitro* studies have shown that a reduction in the GSH content is necessary for the formation of the apoptosome [11]. We demonstrated here that GSH depletion requires initiator caspase activation, while it is independent from executioner caspase, which corroborates our previous observations showing that GSH depletion is a prerequisite for the activation of the execution phase of apoptosis [7]. TNF α -induced apoptosis and GSH depletion have been shown to depend on the activation of interleukin-1 β -converting enzyme-like protease (caspase 1) independent of Bcl-2 [59]. Paradoxically, GSH depletion has also been reported to inhibit rather than stimulate apoptosis [17, 60, 61]. However, recent studies suggest that this might be mediated by transcriptional mechanisms involving the upregulation of antiapoptotic proteins such as Bcl-2, heat shock proteins, and NF- κ B [62, 63] by chronic GSH depletion. Resistance to GSH depletion-induced cell death has been reported to be mediated by activation of Nrf2 that in turn, upregulates other antioxidant systems including catalase and glutathione S-transferases [64].

Interestingly, we also observed that initiator caspase activation/cleavage and loss of MMP were enhanced by stimulation of GSH depletion with MK571 and reduced by prevention of GSH loss. These results are consistent with the notion of a positive feedback loop between GSH depletion and the mitochondrial pathway of apoptosis. Activation of caspases is not a unidirectional event as amplification of the apoptotic signal is mediated in part through positive feedback loops whereby activated executioner caspases can proteolytically cleave initiator caspases to further enhance their activity [65–67]. In addition, it has been reported that caspase 9 cleavage by caspase 3 enhances apoptosis by alleviating its inhibition by the X-linked inhibitor of apoptotic proteases (XIAP) [68]. Furthermore, activation of executioner caspases has been shown to regulate the loss of MMP, Cyt C release [69] and cleavage of pro-apoptotic BID [70]. Caspase 9 and 2 activity, as well as Bcl-2, have been shown to regulate caspase 8 cleavage and MMP loss [25, 71–73]. As reviewed in [74], all caspases undergo proteolysis during activation, which is sufficient for executioner caspases to be converted from the inactive zymogen into an active enzyme. This is due to the fact that executioner caspases form dimers, which can give rise to the heterotetrameric form of enzymatic active caspases. In contrast, inactive initiator caspases are monomers that require homodimerization for their activation, which is facilitated by caspase recruitment to

oligomeric platforms such as the DISC or the apoptosome by adaptor molecules. Initiator caspase recruitment enforces an increase in caspase concentration and generates activity by proximity-induced dimerization. Dimers of initiator caspases like caspase 8 show low-level activity, which is locally restricted (e.g. to the DISC) and which alone does not promote cell death [75]. For apoptosis to proceed, caspase 8 has to be (auto-)proteolytically cleaved, a process which is now considered a maturation event as it greatly stabilizes the caspase-8 catalytic domain, enabling activity to remain in the cytosol after it is released from the DISC [76]. Thus, coordinated dimerization and cleavage of the zymogen seems to be required to produce efficient activation and apoptosis [77]. Our results thus suggest that initiator caspase activity induces GSH depletion and that alteration in GSH loss might contribute to enhance caspase processing/cleavage and protease maturation.

In conclusion, our results (Figure 9) demonstrate that initiator caspase activation is required for GSH depletion during apoptosis induced by both extrinsic and intrinsic pathways. However, GSH loss and its stimulation amplify the initiation phase of apoptosis by regulating caspase processing/activation. Finally we present compelling evidence against the role of MRP1 proteins in GSH loss and apoptosis, which underscores the importance of future research aimed to identify new potential transporters for GSH loss during apoptosis.

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LIST OF ABBREVIATIONS

ABCC1	ATP-binding cassette, subfamily C, member 1
ABCG2	ATP-binding cassette (ABC) transporter, the subfamily G member 2
Apaf1	Apoptotic protease activating factor 1
Bcl-2	B-cell lymphoma 2
BID	BCL-2 Interacting Domain
Caspase	cysteine-aspartic proteases or cysteine-dependent aspartate-directed proteases
CF	5-carboxyfluorescein
CFDA	5-carboxyfluorescein diacetate
C8DF	caspase 8 deficient cells, I9.2 clone
C9DF	caspase 9 deficient cells, JMR clone
C9RE	caspase 9 deficient cells where caspase 9 has been re-introduced
Cyt C	cytochrome C
CFTR	cystic fibrosis transmembrane conductance regulator
DISC	death-inducing signaling complex
DMSO	dimethyl sulfoxide
FACS	Fluorescence Activated Cell Sorting
FADD	Fas-Associated protein with Death Domain
FADDDF	FADD deficient jurkat cells, I2.1 clone

FasL	Fas ligand
FLIP	cellular FLICE-inhibitory protein
GLAST	glutamate/aspartate transporters
GSH	glutathione
GSSG	glutathione disulfide
mBCl	monochlorobimane
MMP	mitochondrial membrane potential
MOMP	mitochondrial outer membrane permeabilization
MRP1	multidrug resistance protein 1
NAC	N-acetyl-L-cysteine
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
OA⁻	organic anions
OATP	organic anion transporting polypeptides
PI	propidium iodide
ROS	reactive oxygen species
RNS	reactive nitrogen species
TNFα	tumor necrosis factor-alpha
UVC	ultraviolet C light
XIAP	X-linked inhibitor of apoptotic proteases

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Figure 1. Changes in GSH content induced by FasL depend on initiator caspase activity
 Changes in intracellular glutathione content [GSH]_i were determined by FACS using the thiol binding dye mBCl. For the induction of apoptosis, Jurkat cells were incubated with Fas ligand (FasL) 4h at the dose indicated (50 ng/ml in A).. Data are expressed as frequency histograms of mBCl fluorescence (A). Cells were pre-incubated for 1 h with Z-VAD-FMK (pan-caspase), Z-IETD-FMK (caspase 8), Z-LEHD-FMK (caspase 9), AC-DMQD-CHO (caspase 3) and AC-DNLD-CHO (caspase 3,7) inhibitors prepared in DMSO and used at a concentration of 50 μM. Controls included DMSO at the same concentration (0.1%). Populations were gated according to their GSH_i levels on mBCl-fluorescence *versus* forward scatter plot [7, 30] and represented as: normal cells with high GSH_i (population in black), and cells with reduced GSH_i levels (population in light grey, and population in grey). In B and C, Jurkat cells and cell lines genetically deficient in caspase-8 (C8DF), FADD

(FADDDF), the corresponding parental wild-type cells (WT, clone A3), cells deficient in caspase 9 (C9DF) and C9DF reconstituted with caspase 9 (C9RE) were analyzed for the expression of pro-apoptotic proteins by WB and FasL-induced GSH depletion was analyzed as explained above (**C** and **E**). Flow cytometry plots are representative of three independent experiments

Figure 2. Knockdown of MRP1 by shRNA correlates with loss of function

Cells were stably transduced with MISSION short hairpin small interference RNA (shRNA) against MRP1 or a non-target shRNA. In **A**, MRP-1 expression was assessed by FACS using FITC-conjugated anti-MRP1 antibody. Jurkat cells express MRP1 observed as an increase in FL-1 fluorescence. Two vectors, 4996-3 and 4996-4, were shown to significantly decrease MRP1 (**B**). MRP1 activity in stable cells overexpressing 4996-3, 4996-4 and non-target shRNA (scramble) was assessed by both CFDA retention (**C**) and release after its cleavage by intracellular esterases (CF) for 4 h in CFDA free medium (**D**). Differences in cell volume in the different cell lines were determined by alterations in forward scatter properties (**C**). Knockdown of MRP1 correlated with increased CFDA retention and decreased CF efflux. * $p < 0.005$ against scramble values

Figure 3. MRP1 knock-down does not affect GSH depletion induced by both extrinsic and intrinsic pathways of apoptosis

Apoptosis was induced in Jurkat cells and Jurkat cells stably overexpressing shRNA against MRP1 (vectors 4996-3 and 4996-4) and non-target shRNA by death-receptor activation (4 h) with FasL (**A** and **C** [50ng/ml]), or by induction of the intrinsic mitochondrial pathway (8 h) via protein synthesis (cycloheximide 30 μ M), topoisomerase II (etoposide 100 μ M), and protein kinase inhibition (staurosporine 20nM), as well as stress (UVC radiation 15mJ/cm²) (**B** and **D** [staurosporine 20nM]). Changes in intracellular GSH were analyzed as explained in Figure 1. Histograms (**A** and **B**) are representative of three independent experiments. Data in **C** and **D** are means $\pm$ SE of n = 3. *p<0.005 against scramble values

Figure 4. MRP1 knock-down does not affect apoptosis induced by FasL

Apoptosis was induced by FasL (4h) in Jurkat cells and Jurkat cells stably overexpressing shRNA against MRP1 (vectors 4996-3 and 4996-4) and non-target shRNA. In **A**, externalization of phosphatidylserine during apoptosis was determined by its recognition with Annexin V conjugated to FITC. Early apoptotic cells (*light grey*) are defined as having Annexin V +/PI -. Late apoptotic and non-viable cells are both Annexin V + / PI + (*dark grey*). Reduction in viable cells upon FasL (50 ng/ml) (**B**) was identified as both Annexin V -/PI- cells (*population in black* in **A**). Contour plots in **A** are representative of 3 independent experiments. Data in **B** are means $\pm$ SE of $n = 3$. * $p < 0.005$ against scramble values. In **C**, Activation of the execution phase of apoptosis was determined by cleavage/activation of executioner caspases 3, 6 and 7 and cleavage/degradation of their substrates PARP, lamin and α -fodrin. Western immunoblot analysis was done on whole cell lysates of experimental samples. Blots were stripped and reprobbed for α -tubulin to verify equal protein loading and are representative of at least 3 independent experiments.

Figure 5. MRP1 knock-down does not affect apoptosis induced by the intrinsic mitochondrial pathway

Apoptosis was induced in Jurkat cells and Jurkat cells stably overexpressing shRNA against MRP1 (vectors 4996-3 and 4996-4) and non-target shRNA by induction of the intrinsic mitochondrial pathway (8 h) with cycloheximide (30 μ M), etoposide (100 μ M), staurosporine (20 nM) or UVC radiation (15 mJ/cm²). Externalization of phosphatidylserine during apoptosis (**A**) was determined as explained in Figure 4. Contour plots are representative of 3 independent experiments. Data in **B** represents loss in cell viability upon staurosporine treatment (20 nM) as in **A** and are means $\pm$ SE of n = 3.

Figure 6. Glutathione efflux regulates the cleavage of initiator caspases 8 and 9 and BID
Apoptosis was induced by 50 ng/ml FasL for the time indicated in the presence or absence of high extracellular GSH medium (A–C) or 50 μ M MK571 (D–F). Western blot analyses were done as explained in Figure 4. Blots are representative of at least 3 independent experiments.

Figure 7. Glutathione efflux regulates the initiator phase of apoptosis through the mitochondrial feedback loop

Apoptosis was induced by FasL (4 h) in the presence or absence of high extracellular GSH medium and/or 50 μ M MK571. In **A** and **F**, cleavage/activation of caspase 8 was measured by FACS in cells immunostained using an anti-cleaved caspase 8 antibody. Loss of mitochondrial membrane potential (MMP) (**B** and **G**) was measured by flow using the JC-1 dual emission. Loss of the MMP results in a reduction in aggregated JC-1 with a concurrent shift to monomeric state. Histograms (**A**) and contour plots (**B**) are representative of 3 independent experiments. Western blot analyses (**C–E**) were done as explained in Figure 4. Blots are representative of at least 3 independent experiments.

Figure 8. GSH depletion regulates the intrinsic mitochondrial pathway of apoptosis

Cells were treated with UVC radiation (30 mJ/cm²) for 4 h in the presence or absence of NAC (10 mM) and/or MK571 (50 μM). Apoptosis, determined by the externalization of phosphatidylserine (**A**) and activation/cleavage of executioner caspases (3,6,7) and their substrates (PARP and α-fodrin) (**C**) and GSH loss (**D**), were determined as explained in Figures 1 and 4. Caspases 3/7 like activity (**B**) was determined using the CaspaTag assay as explained in materials and methods. Caspase 3/7 activity is observed as an increase in FL1-fluorescence that precedes the loss of plasma membrane activity detected by PI uptake. Contour plots and blots are representative of at least three independent experiments.

Figure 9. Role of GSH depletion in both extrinsic and intrinsic signaling cascades

We previously demonstrated that GSH depletion is required for the execution phase of apoptosis (1) [7]. In this work we report that GSH depletion depends on the activity of initiator caspases 8 and 9 (2). Interestingly, GSH depletion and its stimulation enhance initiator caspase cleavage (*broken lines*) during apoptosis induced by both extrinsic and intrinsic pathways. This is explained by the fact that caspase activation is not a unidirectional event and that in type II cells (Jurkat), death-receptor pathway relies on the amplification of the signaling cascades by BID activation and translocation to the mitochondria (3). Finally we present compelling evidence that MRP1 transporters are not involved in GSH depletion and apoptosis induced by both extrinsic and intrinsic pathways as MRP1 inhibitors and other structurally unrelated OA⁻ stimulate GSH loss (4) [7]. GSH-T (putative GSH transporter), OA⁻ (organic anions such as MK571), Cyt C (cytochrome C), FADD (Fas-Associated protein with Death Domain), BID (Bcl-2 Interacting Domain), BH3 (BH3 only proteins such as BID and BIM which induce oligomerization of Bax or Bak and release of Cyt c)