Resistance to Fas-induced apoptosis in hepatocytes: role of GSH depletion by cell isolation and culture

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Musallam, Lina, Chantal Éthier, Pierre Selim Haddad, Francine Denizeau, and Marc Bilodeau. Resistance to Fas-induced apoptosis in hepatocytes: role of GSH depletion by cell isolation and culture. Am J Physiol Gastrointest Liver Physiol 283: G709–G718, 2002. First published May 15, 2002; 10.1152/ajpgi.00013.2002.—The involvement of reduction/oxidation (redox) state in cell sensitivity to apoptosis has been suggested by several studies in which induction of apoptosis was shown to require oxidative stress or GSH extraction. On the other hand, biochemical studies of caspases revealed that their activation necessitates a reduced cysteine in their active site. This is ensured by maintaining intact intracellular glutathione status during apoptotic induction as reported by in vivo studies. Therefore, we investigated the relationship between intracellular glutathione levels and the sensitivity of mouse hepatocytes in culture to Fas-induced apoptosis as well as potential mechanisms responsible for this sensitivity. We found that total and reduced glutathione levels are decreased by one-half during cell isolation procedure and further decline by 25% during cell culture for 2 h in normal Williams’ E medium. Cell culture in medium supplemented with cysteine and methionine maintains glutathione at a level similar to that measured just after cell isolation. Results show that the capacity of Fas to activate caspase-8 and to induce apoptosis requires important intracellular glutathione levels and high GSH/total glutathione ratio. In conclusion, the present study shows that intracellular glutathione plays an important role in maintaining the apoptotic machinery functional and is thus capable of transmitting the apoptotic signal.

Oxidative stress is defined as the manifestations of cell or tissue following exposure to excess oxidants (20). Reactive oxygen species (ROS), such as O$_2^·$, OH, and H$_2$O$_2$, are the principal species of intracellular oxidants. They are generated as by-products of electron transport through the mitochondrial respiratory chain as well as by γ-ray and ultraviolet light irradiations (20, 27). ROS are highly reactive toward intracellular macromolecules (DNA, proteins, and lipids), causing severe lesions that can lead to cell death by either necrosis or apoptosis, depending on the intensity of the oxidative stimuli (24, 40, 41).

Hence, it is necessary for cells to tightly control their content in ROS. To do so, cells have developed several antioxidant (or reductant) mechanisms that maintain the intracellular redox environment in a highly reduced state. These mechanisms range from scavengers (e.g., glutathione, vitamins C and E) to enzymes that neutralize ROS (e.g., superoxide dismutase, catalases, and glutathione peroxidase) before they could exert any damage (20, 27). Glutathione, a tripeptide (L-glutamyl-L-cysteinyl-glycine) synthesized in the cytoplasm, is the most abundant intracellular nonprotein thiol involved in antioxidant elimination. It is used by different enzymes to reduce not only ROS but oxidized macromolecules as well (20, 28). Several oxidative injuries have been associated with glutathione depletion (1, 9, 19, 20). That is why glutathione replenishment by N-acetylcysteine administration has been proposed as a therapeutic strategy for oxidative stress injuries (9).

Apoptosis can be triggered by different stimuli, including cell surface receptors [e.g., tumor necrosis factor (TNF)-α and Fas (10, 26, 33)], γ-irradiation (30), staurosporine (39), and others. These stimuli converge onto common intracellular effectors such as caspases, endonucleases, and proapoptotic proteins of the BCL-2 family (e.g., Bad, Bid, Bax). The Fas apoptotic pathway was recently elucidated. Binding of Fas ligand to Fas receptor (Fas-R) results in receptor trimerization leading to the recruitment of the adapter protein FADD to the death domain of Fas-R (11). The death effector domain of FADD can then interact with a similar domain of procaspase-8, which results in the oligomerization of the latter. Activation of caspase-8 through autocleavage leads to a series of downstream events, including Bid cleavage, cytochrome c release from the mitochondria, and caspase-3 activation, finally culminating in cell death by apoptosis. (43) Ogasawara et al. (36) reported that intraperitoneal administration of anti-Fas antibodies causes severe liver damage by apoptosis within 1–2 h, leading to rapid animal demise.
within 6 h. On the other hand, it is well documented that induction of apoptosis in cultured hepatocytes is less successful (23, 34).

As mentioned above, it was recently reported that apoptosis could be triggered by weak oxidative stimuli. In these studies, antioxidant depletion sensitized cells to apoptosis, whereas their replenishment abrogated it (19, 24, 41). Moreover, antioxidant extrusion (such as glutathione) was reported as an early and requisite event in the mediation of the apoptotic signal (13, 42). However, this remains controversial. Recent reports have shown that, on the contrary, oxidants prevented cell death by apoptosis. For example, caspases, which play a pivotal role in apoptosis, are themselves redox sensitive. They require a reduced cysteine in their active site to function (25, 35). In parallel, Hentze et al. (16, 17) reported that intact intracellular glutathione status is essential for receptor-mediated caspase activation and apoptosis in vivo.

Therefore, in light of the importance of glutathione for Fas-induced apoptosis in vivo, we investigated the possible relationship between glutathione status and the resistance of cultured mouse hepatocyte to Fas-induced apoptosis as well as the potential mechanisms involved in this resistance. We found glutathione status to be important for the ability of Fas to induce apoptosis in culture. This resistance in culture appears to result from a lower capability to induce the proteolytic activity of caspase-8.

**MATERIALS AND METHODS**

All animals received humane care according to the guidelines of the Canadian Council on Animal Care. Experimental protocols were approved by the Comité institutionnel de protection des animaux of the CHUM-Hôpital Saint-Luc.

**Hepatocyte Isolation and Culture**

Hepatocytes were isolated from the liver of fed male BALB/c mice (22–25 g) using the two-step collagenase perfusion method described previously (32). Cells were seeded onto plastic Petri dishes (26,000 cells/cm²) in medium supplemented with 10% fetal bovine serum (GIBCO BRL, Toronto, ON, Canada) and allowed 2 h to attach. Cysteine (Cys; the limiting amino acid for glutathione synthesis) and its precursor methionine (Met) were used to modulate glutathione intracellular levels. Therefore, we used two different culture media: 1) normal Williams’ E medium (GIBCO BRL) containing 40 mg/l Cys and 15 mg/l Met (medium N) and 2) Williams’ E medium supplemented with Cys (90 mg/l) and Met (medium C + M; 55 mg/l). The serum-containing medium was removed after attachment, and cells were incubated with serum-free medium for the indicated times in each experimental series. Apoptosis was induced in experimental groups with mouse anti-Fas Jo2 antibody (Research Diagnostics, Flanders, NJ) at a concentration of 100 ng/ml, unless stated otherwise, either directly after attachment in medium N and C + M or after 6 h of cell incubation in medium C + M.

**Determination of Total and Reduced Glutathione Levels**

**Culture conditions.** To determine the effect of hepatic isolation procedure on total (GSx) and reduced (GSH) glutathione, we measured GSx and GSH levels at different stages of the isolation procedure. First, to measure glutathione levels in total liver, a section of the right lobe was homogenized in 0.25 M sucrose solution (20% wt/vol). Cells (2 × 10⁶) were also collected after liver perfusion with HEPES and collagenase solutions, as well as after hepatocyte purification, and washed once in cold PBS before being resuspended in 300 μl of 0.25 M sucrose solution. To evaluate glutathione levels over time in culture, hepatocytes were attached in either medium N or C + M in the presence of 10% fetal bovine serum for 2 h. Then, the serum-containing medium was removed, and cells were cultured with medium C + M for 6 h. At the end of the experiment, cells were scraped off, pelleted by centrifugation, and resuspended in 300 μl 0.25 M sucrose solution. Samples were stored at −80°C until determination of GSx and GSH levels.

**GSx and GSH determination.** GSx and GSH levels were measured, as described previously (31), with some modifications. Briefly, samples were boiled for 10 min, then centrifuged at 12,000 g for 15 min at room temperature. The pellet was discarded. A portion of the supernatant (100 μl) was treated with glutathione reductase (1.2 U; Sigma, Oakville, Canada) and NADPH (1.2 mM; Sigma) for 10 min at room temperature. The reaction was stopped by precipitating proteins with 4% sulfosalicylic acid (1:1; Sigma). GSH was measured directly in the supernatant after protein precipitation. Thiol concentration was determined by adding DTNB (0.01 M; Sigma) to the sample at a 9:1 dilution and measuring the absorbance at 412 nm. Protein concentration in each sample was determined according to Bradford (5).

**Morphological Determination of Apoptosis**

Cells were incubated after attachment with medium N or C + M alone or in the presence of anti-Fas antibodies. After 6 h, the medium was removed and cells were fixed with 5% formaldehyde solution (Anachemia Science, Lachine, Canada) and then stained with Hoechst 33258 (250 ng/ml; Sigma) to quantify apoptosis, as described previously (32). The percentage of apoptotic cells is expressed as the ratio of apoptotic nuclei to the total number of nuclei (normal + apoptotic). We evaluated 400 nuclei for each Petri dish.

**Biochemical Determination of Cell Death**

Cells cultured in medium C + M were induced to undergo apoptosis with anti-Fas antibodies directly after attachment or 6 h after attachment. To determine cell death, we measured aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels released in the medium after 24 h in culture, as detailed elsewhere (32). AST or ALT level for each sample was calculated as the ratio of AST or ALT present in the medium to the sum of the levels of AST or ALT released in the medium and that present in the homogenate of adherent cells. Results are presented as the percentage of AST or ALT level released in Fas-treated cultures to that released in untreated cultures.

**Measurement of Caspase-8 Activity**

**Culture conditions.** To determine the effect of medium on Fas-induced caspase-8 activity, cells were treated, after attachment, with anti-Fas antibodies (250 ng/ml) for 3 h in medium N or C + M. In parallel, the effect of time in culture was evaluated on cultures incubated with anti-Fas antibodies (for 3 h) either directly after attachment or 6 h later in medium C + M. Cells were then scraped off in PBS and collected by centrifugation. Cells were lysed for 15 min on ice...
in lysis buffer (10 mM HEPES (pH 7.4), 5 mM MgCl₂, 42 mM KCl, 0.1 mM EDTA, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO; Calbiochem; San Diego, CA), 0.1% Triton X-100, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml soybean trypsin inhibitor, and 100 µM benzamidine). Lysates were centrifuged at 13,000 g for 10 min, and the supernatant was stored at −80°C. Human activated recombinant caspase-8 (Calbiochem) was used to determine the glutathione-dependent modulation of caspase-8 activity. To do so, activated caspase-8 (30 U/well) was mixed with assay buffer containing 5 mM GSx with different ratios of GSH/GSx (100 to 0%).

**Measurement of the proteolytic activity.** The fluorometric Ac-IETD-AMC cleavage assay was performed in microtiter 96-well plates. Reaction mixture contained 40 µl lysates (200 µg protein) and 50 µl assay buffer [2×, 100 mM HEPES (pH 7.2), 200 mM NaCl, 2 mM EDTA, 20% sucrose, 0.2% CHAPSO, 20 mM dithiothreitol (DTT)]. After an initial 10-min incubation at 37°C, the reaction was started by adding 10 µl of caspase-8 fluorescent substrate Ac-IETD-AMC (100 µM; Biosource International, Camarillo, CA). The cleavage activity of caspase-8 was evaluated over a period of 30 min using 380 and 460 nm as excitation and emission wavelengths, respectively in a SPECTRAmax GEMINI microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA). The maximal substrate cleavage rate (V_{max/s}) was calculated by SOFTmax Pro software (Molecular Devices). The activity of caspase-8, presented in units, was derived from a calibration curve relating V_{max/s} to increasing units of human activated recombinant caspase-8.

**Immunoblotting**

**Cell lysis.** We determined the expression level of procaspase-8, Bid, and BCL-xL proteins after 0, 0.5, 1, and 3 h postcell attachment. At the end of the experiment, the medium was removed and cells were then scraped off and pelleted by centrifugation. Cells were subsequently disrupted by sonication (Sonic & Materials, Danbury, CT) in the presence of lysis buffer (PBS, pH 7.4; 1% Igepal CA-630; 0.5% deoxycholic acid sodium salt; 0.1% SDS; 5 mM EDTA; 10 µg/ml leupeptin; 10 µg/ml aprotinin; 10 µg/ml soybean trypsin inhibitor; and 100 µM benzamidine).

**Western blot.** Proteins samples (125 µg) of each experimental condition were separated by electrophoresis on 15% SDS-polyacrylamide gels (38). Equal protein loading was assessed by staining the membranes with Ponceau S (Sigma). Blots were probed with primary antibodies for 2 h, then with secondary antibodies for 1 h, both at room temperature with gentle agitation. The expression of procaspase-8 (57 kDa), Bid (23 kDa), BCL-xL (29 kDa), and actin (42 kDa) proteins were detected with rabbit anti-human caspase-8 (1 µg/ml; R&D Systems; Minneapolis, MN), goat anti-human/mouse Bid (1 µg/ml; R&D Systems), mouse monoclonal anti-BCL-x antibody (0.5 µg/ml; Transduction Laboratories, Lexington, KY), and anti-mouse actin monoclonal IgM ascites (1:2,000; Oncogene Research Products; Cambridge, MA), respectively. Peroxidase-conjugated anti-mouse IgG (1:10,000; BD Pharmingen; Mississauga, CA), peroxidase-conjugated anti-rabbit IgG (1:10,000; Santa Cruz Biotechnology), and peroxidase-conjugated anti-mouse IgM (1:10,000; Oncogene Research Products) activities were revealed using Western Blot Chemiluminescence Reagent Plus (NEN Life Science Products, Boston, MA). Blots were then scanned, and bands were quantified by densitometry.

**Statistical Analysis**

All data represent the values of at least five experiments, each from different cell isolation. Differences between groups were analyzed by one-way ANOVA for repeated measures, unless otherwise stated. The difference between treatment and time was analyzed by two-way ANOVA for repeated measures. A P value <0.05 was considered significant.

**RESULTS**

**Cell Isolation and Culture Decrease Liver GSx Levels**

As mentioned above, glutathione is a very abundant intracellular thiol in the liver. GSx concentration amounted to 2.04 ± 0.33 nmol/µg protein in total liver (Fig. 1). Levels were greatly diminished during the cell isolation procedure. Indeed, GSx levels were decreased by 46% following liver digestion with collagenase (1.11 ± 0.09 nmol/µg protein; P < 0.01) but were not significantly reduced by hepatocyte purification (1.01 ± 0.09 nmol/µg protein; not significant (NS)). When cells were cultured for 2 h in commercial Williams' E medium (medium N), GSx levels were further diminished by 46% (0.55 ± 0.04 nmol/µg protein; P < 0.01) compared with just before culture. This signifies that, after 2 h of attachment, cells retained only 27% of their original content in GSx (P < 0.001).

**Cys and Met Supplementation Stabilizes GSx Levels in Cultured Hepatocytes**

To modulate GSx synthesis, we added Cys and Met to the culture medium at a final concentration of 90 and 55 mg/l, respectively (medium C + M). These levels were stabilized by 46% (1.01 ± 0.09 nmol/µg protein; P < 0.01) compared with medium N levels. As shown in Fig. 1, at the end of the 2-h incubation, cells retained only 27% of their original content in GSx (P < 0.001).

**Fig. 1.** Effect of cell isolation and culture on total glutathione (GSx) levels. GSx levels were measured, as described in MATERIALS AND METHODS, in total liver (right lobe), after liver perfusion with collagenase, in purified hepatocytes and after 2 h attachment in serum-containing commercial Williams’ E medium (medium N) or Williams’ E medium supplemented with Cys and Met (medium C + M). The data are presented as nanomoles of GSx per microgram of proteins collected after sample preparation for glutathione determination. Values are depicted as means ± SE from 5 experiments. **P < 0.01; ***P < 0.001 compared with total liver; #P < 0.01.
concentrations were chosen by performing a dose-response curve where we determined the effect of different concentrations of these two amino acids on intracellular GSx levels. As shown in Fig. 1, cell culture in this enriched medium prevented the loss of GSx that was observed in medium N during the 2-h attachment (0.99 ± 0.17 vs. 0.55 ± 0.043 nmol/μg protein for medium C + M vs. N; P < 0.01).

Of note, addition of greater concentrations (up to 120 mg/l Cys and 75 mg/l Met) of these two amino acids to the culture medium during cell attachment resulted in 50% restoration of GSx that was lost during liver digestion. However, it was more rapidly evacuated from the cells in culture compared with medium C + M (results not shown). Thus our experimental conditions were optimal in preserving GSx in cultured mouse hepatocytes.

**Ability of Fas to Induce Apoptosis is Greater in Cells Cultured in Medium C + M**

Our untreated cultures displayed very low rates of apoptosis (<1%), with no significant difference between the two media (data not shown). When hepatocytes were incubated with an agonistic Fas-R antibody, a significant apoptotic response was observed in both media. Of note, the level of necrosis, as measured by trypan blue exclusion, was not significantly different between untreated and Fas-treated cultures or between the two media (data not shown). Interestingly, however, the level of apoptotic cells, as determined by condensed chromatin fluorescence, was more than twice as great in medium C + M (10.0 ± 0.9%) as in medium N (4.4 ± 0.2%; P < 0.05; Fig. 2A). Similarly, Fas-induced AST release was higher in medium C + M (127.6 ± 3.1%) compared with medium N (114.6 ± 4.0%; P < 0.001; Fig. 2B). In addition, Fas-induced release of ALT was significantly higher in medium C + M (209.6 ± 9.3%) compared with medium N (186.0 ± 10.4%; P < 0.05; Fig. 2C).

**Expression Levels of Procaspase-8, Bid, and BCL-xL Proteins are Similar in Both Media Over Time in Culture**

Apoptotic induction depends on the ratio of anti- and proapoptotic regulating proteins. Caspase-8, Bid, and BCL-xL are some of the key proteins implicated in the regulation of Fas pathway in hepatocytes. Therefore, we measured the level of expression of procaspase-8 (57 kDa), Bid (23 kDa), and BCL-xL (29 kDa) in both media for up to 3 h postattachment by Western blot analysis. Figure 3 shows that protein expression of procaspase-8 (Fig. 3A), Bid (Fig. 3B), and BCL-xL (Fig. 3C) in medium N was not significantly different from that in medium C + M (NS) and this at each time point tested. This suggests that the difference in the ability of Fas to induce cell death is not due to different expression levels of these key apoptosis-regulating proteins.

**Fas Capacity to Activate Caspase-8 Proteolytic Activity is Greater in Medium C + M**

Because the expression level of procaspase-8 was similar in cultures incubated with either medium N or C + M, we next examined the protease activity of caspase-8 in untreated cultures or in cells treated with anti-Fas antibodies for 3 h directly after cell attachment in both media N and C + M (Fig. 4). In untreated cultures, levels of caspase-8 activity in both media N (1.16 ± 0.45 U) and C + M (1.41 ± 0.25 U; NS) were
low. After 3 h of Fas receptor stimulation, the activity increased to 8.70 ± 1.25 and 13.60 ± 0.79 U in media N and C/M, respectively (P < 0.001), with the difference between the two media being significant (P < 0.05).

**GSH/GSx Ratio Declines Over Time in Culture**

Normally, GSH/GSx ratio is kept stable in cells by different mechanisms, including GSSG reduction and GSSG active extrusion. This ratio was at 83.0 ± 7.3% in total liver and was not significantly modified during liver perfusion, hepatocyte purification, or cell attachment for 2 h (results not shown). However, GSH/GSx ratio was observed to decrease in our cultures over time. Indeed, hepatocellular content in GSx was decreased by 25% after 6 h culture with medium C/M compared with just after 2 h attachment (P < 0.05; Fig. 5A). Nonetheless, GSx levels were 60% higher in medium C/M than in medium N at 6 h (P < 0.05; data not shown). On the other hand, GSH levels were decreased from 27.65 ± 2.61 to 16.95 ± 3.31 nmol/10^6 hepatocytes (P < 0.05), which represents a 39% reduction (Fig. 5A). This translates into a significant decline of GSH/GSx ratio from 82.0 ± 2.6% at 2 h attachment to 65.3 ± 5.9% at 6 h (P < 0.05; Fig. 5B). Although a similar decrease of GSH/GSx ratio was observed in cells cultured in medium N (data not shown), it remained significantly higher in medium C/M (74.0 ± 2.1%) compared with medium N (67.0 ± 2.6%; P < 0.05) when measured at 3 h postattachment.

**GSH/GSx Ratio Decline is Associated With a Decrease in Fas-Induced Caspase-8 Activation and Cell Death**

It is well documented that profound GSH loss could promote oxidative stress, which subsequently leads to cell death. However, because we observed lower levels of Fas-induced apoptosis when GSx levels were de-
completed, we investigated whether the observed decrease in GSH/GSx ratio (17% drop) over time in culture was also able to reduce the transmission of Fas signal. Therefore, apoptosis was induced after 6 h of culture, and caspase-8 activity as well as cell death were then assessed. As illustrated in Fig. 6A, there was an 18% decline in the proteolytic activity of caspase-8 from 13.60 ± 0.79 (at 2 h attachment) to 11.09 ± 0.71 U (after 6 h culture; *P < 0.05). This decline occurred concomitantly with a decrease in the capacity of Fas to induce cell death (Fig. 6, B and C). Indeed, AST released from cultures incubated with Fas antibodies after 6 h of attachment was 19% less (113.3 ± 4.0%; *P < 0.01) than those incubated shortly after attachment (after 30 min; 140.0 ± 6.0%; *P < 0.01). Similarly, ALT release was decreased by 23% in a time-dependent manner as shown in Fig. 6C (30 min: 235.4 ± 10.6% vs. 6 h: 204.5 ± 10.2%; **P < 0.01).

To determine which form of GSx is associated with the observed decrease in GSH/GSx ratio (17% drop) over time in culture, we established a correlation between the levels of GSH and GSH/GSx ratio on one hand and ALT levels on the other. To do so, we conducted separate sets of experiments, in which cells were incubated with anti-Fas antibodies after different periods of culture in medium C + M following attachment, whereas other cells from the same animal served to measure GSH and GSx levels at the same time points. There was a significant correlation between ALT levels released and intracellular GSH levels (*R² = 0.476; *P < 0.05). However, the correlation was much more important between GSH/GSx ratio and ALT levels (*R² = 0.846; **P < 0.0001). This suggests that relative accumulation of GSSG in cells is associated with a...
reduction in the capacity of Fas to induce apoptosis over time in culture.

Recombinant Caspase-8 Activity is Decreased When GSH/GSx Ratio is Diminished In Vitro

We next investigated GSx-dependent modulation of caspase-8 by determining the effect of varying GSH/GSx ratios on the proteolytic activity of the activated recombinant human form of this enzyme (Fig. 7). Caspase-8 activity was not significantly different between 100 and 75% GSH/GSx ratio (20.61 ± 1.55 and 19.72 ± 0.70 U, respectively, NS). Conversely, the activity of the protease significantly decreased when this ratio declined to <75% (R² = 0.93; P < 0.01). When 100% GSSG (i.e., 0% GSH/GSx ratio) was attained, there was no caspase activity detected.

DISCUSSION

The involvement of oxidative stress in cell death by necrosis is well established. Indeed, many studies have demonstrated the devastating effects of oxidants on intracellular macromolecules. For example, ROS have been shown to inactivate vital proteins (such as glutathione peroxidase and adenyl cyclase) and cause DNA strand breaks and membrane lipid peroxidation (20, 27). As a result, cytosolic Ca²⁺ is increased, ATP is depleted, NADPH and GSH are oxidized, and membrane integrity and signaling are impaired (37). All these lesions are very harmful if not repaired. In a prolonged exposure to oxidants, cells exhaust their antioxidant mechanisms and their repair systems are overwhelmed, leading to cumulative lesions and, therefore, to necrosis (20, 37).

In apoptosis, however, the question is still under debate. On one hand, depletion of reductant or antioxidant reserve was found to sensitize otherwise unresponsive cells to apoptosis. This was observed in several cell lines, including Jurkat (42), HepG2 (13), and cholangiocytes (6). In these studies, depletion of glutathione accentuated the apoptotic response, whereas high intracellular glutathione levels abrogated it. In addition, low amounts of oxidants, such as H₂O₂, were found to induce apoptosis in HL-60 cells (24). On the other hand, growing evidence demonstrates that oxidants may actually prevent apoptosis. For example, nitric oxide-induced apoptosis of macrophages (4), Fas-induced apoptosis of T cells (14), and cytokine-mediated apoptosis of hepatocytes in vivo (18) were found to depend on sufficient intracellular glutathione levels. The redox sensitivity of caspases was hypothesized to be responsible for the observed protection because of decreased glutathione levels. In parallel, hepatocyte treatment with O₂⁻ was shown to prevent Fas-induced apoptosis (8). Therefore, it still remains unclear whether oxidants are pro- or antiapoptotic. The aim of this study was to determine the involvement of the major intracellular antioxidant system glutathione in the sensitivity of primary hepatocyte cultures to Fas-induced apoptosis.

Because glutathione is known to be very abundant in the liver (9), we wanted to evaluate the effect of the cell isolation and culture procedure on GSx levels before determining the effect of glutathione on apoptosis in culture. Our data show important GSx depletion as a result of cell isolation and culture. Indeed, isolated hepatocytes cultured in commercial Williams’ E medium (medium N) for 2 h retained only 27% of their original content of GSx. Medium supplementation with Cys and Met, although not fully restoring GSx levels to those in total liver, prevented GSx loss due to cell culture. In addition, this enriched medium maintained more stable GSx levels during extended cell culture as opposed to medium N, because these levels remained twice higher in medium C + M than in medium N after 6 h of culture in serum-free conditions (data not shown).

GSx represents the sum of cellular content in GSH and GSSG. Because the GSH/GSx ratio was identical in cells cultured in either medium (after 2 h attachment), the difference in GSx levels most likely signifies that both GSH and GSSG levels diminished. Several studies have reported that cells can lose GSH by active extrusion and passive diffusion in addition to its utilization as a metabolic cofactor (9). In fact, GSH efflux from hepatocytes is the first step in the degradation of hepatic glutathione (9). Therefore, glutathione levels can drop drastically if not compensated. In parallel, the supply of the three amino acids that constitute glutathione is limited in the context of cell culture. Hence, glutathione synthesis is expected to decrease over time in culture. Concerning GSSG, cells convert it to GSH under normal redox state (>80% of GSx being in the form of GSH) and increase its extrusion under oxidative stress (9). Therefore, normal culture conditions will result in a net loss of GSH and GSSG, i.e., GSx.

When Cys and Met were added to the culture medium (medium C + M), Cys supply was increased both directly and indirectly, Met being a precursor of Cys. This ensures continued glutathione synthesis. In addition, the presence of Met on the external surface of the...
hepatocyte has been shown to inhibit GSH efflux from isolated hepatocytes (2, 3). Therefore, the stabilization of GSx levels in cell cultures by medium C + M probably reflects the maintenance of glutathione synthesis and the attenuation of GSH efflux.

On the other hand, GSH/GSx ratio, which remained stable during cell isolation and attachment, declined (17% drop) over an extended period of culture in a similar manner in media N and C + M. This drop is indicative of relative GSSG accumulation in the cells. Therefore, it seems that the processes or mechanisms responsible for GSSG elimination (reduction to GSH or active GSSG extrusion) lose their efficacy in time. Normally, the rapid reduction of GSSG to GSH is catalyzed by GSSG reductase, which requires NADPH as a cofactor (1, 28). It could be postulated that alteration of NADPH availability may affect GSH regeneration and, consequently, promote GSSG accumulation in the cell. Such factors could participate in the observed decrease of the GSH/GSx ratio as a function of culture time.

After the establishment of changes in GSx and GSH/GSx ratio in isolated hepatocytes in culture, we next evaluated the capacity of Fas to induce apoptosis in mouse hepatocytes cultured in medium N vs. C + M. Our results showed that cells cultured in medium C + M were more sensitive to Fas-induced apoptosis than congeners kept in medium N. Indeed, after Fas-receptor stimulation, there was a higher proportion of apoptotic cells and increased AST and ALT release from cells cultured in medium C + M compared with those cultured in medium N. This higher sensitivity toward Fas-induced apoptosis was not due to differential expression levels of procaspase-8, Bid, or BCL-xL proteins, some of the key proteins regulating the Fas apoptotic pathway. Rather, it seems to result from the higher Fas-induced proteolytic activity of caspase-8 observed in cells cultured in medium C + M compared with medium N. In particular, it seems that equivalent proportion of activated caspases exhibit a more robust catalytic capacity in high GSH/GSx conditions. Indeed, our in vitro study on fixed amounts of recombinant activated caspase-8 clearly demonstrates the negative effect of oxidant (GSSG) on the proteolytic activity of this enzyme. In parallel, we observed a higher GSH/GSx ratio in medium C + M compared with medium N. This reinforces the hypothesis that lowering cells’ buffering capacity, by GSH depletion and/or GSSG accumulation, reduces cell sensitivity toward Fas-induced apoptosis.

As mentioned above, oxidative stress is closely associated with cell death and necrosis. Consequently, replenishment of antioxidants, including glutathione, has been repeatedly shown to protect against cell death. Hence, it seems contradictory to find that better preservation of glutathione levels is associated with increased sensitivity toward apoptosis. However, it is important to point out that the liver is naturally sensitive to apoptosis. Indeed, Fas is capable of inducing massive and rapid apoptosis in vivo even in the presence of high intracellular concentrations of glutathione (17, 36). This means that apoptotic signal transduction and effectors are functional in a reducing environment. In parallel, it is well documented that hepatocytes in culture acquire resistance against apoptosis (23, 34). Because there was a drastic decrease of glutathione levels during cell isolation and culture, this suggests that the decrease in glutathione levels is responsible for the culture-acquired resistance to apoptosis. This hypothesis is further supported by the strong positive correlation between GSH/GSx ratio and Fas-induced apoptosis. In addition, caspase-8 proteolytic activity, from both human recombinant and cell lysate sources, directly and significantly correlated with GSH/GSx ratio. A similar relationship was reported by Hentze et al. (17) for recombinant activated caspase-3.

Conclusions similar to the present work have been reached in studies conducted by the groups of Wendel (16, 17), Jäeschke (22), and Pessayre (15). These studies showed that in vivo depletion of glutathione, whether by acetaminophen or phorone administrations, protected the liver against receptor-induced apoptosis. Collectively, these data therefore lend support to the concept that GSH depletion and/or relative GSSG accumulation, which lower the cell’s capacity to buffer against endogenous oxidants, reduce cell sensitivity toward receptor-mediated apoptosis. Furthermore, results from the present study offer diminished caspase-8 activity, the apex of Fas/TNF-α receptor apoptotic signal, as an explanation for the acquisition of resistance toward Fas-induced apoptosis by hepatocytes in culture compared with in vivo conditions.

It is noteworthy that GSH depletion reported in this study occurred very rapidly. This has an importance at two levels. First, it is well documented that GSH levels play a major role in mitochondrial function. In fact, mitochondrial GSH/GSSG ratio is a known modulator of the mega-channel complex, which is responsible for the mitochondrial transmembrane potential (ΔΨm) (7, 12, 21). Therefore, GSH depletion should affect mitochondrial function and, subsequently, effectors of the apoptotic pathway. However, mitochondria retain GSH far more efficiently than the cytoplasm. Indeed, depletion of GSH from the cytoplasm may occur very rapidly (t1/2 = 2 h; see Fig. 1 and Ref. 29). On the other hand, it takes >30 h for the mitochondria to lose one-half of its GSH content (t1/2 = 30 h), even in conditions of extreme GSH depletion (29). Therefore, because of the rapid loss of GSH in our experimental conditions (during isolation procedure and short-term culture), there is potentially not enough time for the mitochondrial pool of GSH to be affected. This leads us to postulate that the effect of GSH on Fas-apoptotic pathway is, in fact, exerted on cytosolic components already present at the time of apoptotic induction. Thus the rapid loss of GSH inhibits some of the key elements in the apoptotic machinery leading to protection against apoptosis. Second, Hauoui et al. (15) recently demonstrated that there is a difference in the sensitivity of the liver toward apoptosis, depending on the length of liver exposure to GSH depletion. Indeed, the authors reported that long-term GSH depletion accentuated apop-
tosis, whereas acute depletion prevented it. It seems that other mechanisms enter into effect to compensate for the loss of cell sensitivity toward apoptosis due to the rapid loss of GSH. Some of these compensatory mechanisms are increased p53 and Bax protein expressions, which are well-known proapoptotic modulators. Our results therefore provide an explanation for the acute effects of GSH depletion on the sensitivity of hepatocytes toward apoptosis. The activity of caspase-8 in situations of chronic GSH depletion would deserve to be evaluated.

In conclusion, our results clearly demonstrate that glutathione depletion by the isolation procedure and cell culture is greatly associated with the culture-acquired resistance to apoptosis. This decrease is related to lower proteolytic activity of caspase-8, which is at the apex of the Fas apoptotic pathway. Therefore, our results confirm the importance of stabilization of intracellular GSx levels for normal cellular function, one of these being the ability of cells to respond to death signals.

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