

Bcl-2-dependent oxidation of pyruvate dehydrogenase-E2, a primary biliary cirrhosis autoantigen, during apoptosis

See related Commentary on pages 187–188.

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The close association between autoantibodies against pyruvate dehydrogenase-E2 (PDC-E2), a ubiquitous mitochondrial protein, and primary biliary cirrhosis (PBC) is unexplained. Many autoantigens are selectively modified during apoptosis, which has focused attention on apoptotic cells as a potential source of “neo-antigens” responsible for activating autoreactive lymphocytes. Since increased apoptosis of bile duct epithelial cells (cholangiocytes) is evident in patients with PBC, we evaluated the effect of apoptosis on PDC-E2. Autoantibody recognition of PDC-E2 by immunofluorescence persisted in apoptotic cholangiocytes and appeared unchanged by immunoblot analysis. PDC-E2 was neither cleaved by caspases nor concentrated into surface blebs in apoptotic cells. In other cell types, autoantibody recognition of PDC-E2, as assessed by immunofluorescence, was abrogated after apoptosis, although expression levels of PDC-E2 appeared unchanged when examined by immunoblot analysis. Both overexpression of Bcl-2 and depletion of glutathione before inducing apoptosis prevented this loss of autoantibody recognition, suggesting that glutathiolation, rather than degradation or loss, of PDC-E2 was responsible for the loss of immunofluorescence signal. We postulate that apoptotic cholangiocytes, unlike other apoptotic cell types, are a potential source of immunogenic PDC-E2 in patients with PBC.

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Introduction

Primary biliary cirrhosis (PBC) is a slowly progressive liver disease characterized by the chronic nonsuppurative destruction of intrahepatic bile duct epithelial cells (cholangiocytes) and high titers of IgG anti-mitochondrial Ab's (1). Although it is an uncommon disease, PBC is a leading indication for liver transplantation among women. Approximately 70% of patients also have salivary gland involvement (2). The only widely used medical treatment, ursodeoxycholate (UDCA), is only moderately effective in preventing progression to cirrhosis (3–5). Interestingly, Gershwin and others (6–8) have determined that over 90% of patients with PBC produce autoantibodies specific for a conformation-dependent epitope of the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2), a ubiquitous mitochondrial matrix protein associated with the inner mitochondrial membrane. Autoreactive T cells specific for PDC-E2 self-peptides have also been isolated from patients with PBC (9, 10). High-titer anti-PDC-E2 autoantibodies with the same specificity are rarely seen in other autoimmune diseases, nor in unaffected relatives of patients with PBC (11). Understanding why an immune response against

this particular autoantigen is so closely associated with PBC may provide insight into the pathogenesis of PBC.

As a group, autoantigens have no common cellular distribution or function that distinguishes them from nonautoantigens. However, a high percentage of autoantigens are specifically cleaved by caspases, apoptosis-specific cysteine proteases (12, 13), and become concentrated in cytoplasmic surface blebs or apoptotic bodies during apoptosis (14). Other autoantigens are phosphorylated or otherwise modified during apoptosis (15). Recent studies suggest that under normal conditions, apoptotic cells engulfed by dendritic cells serve as a source of self-antigens for the induction of peripheral self-tolerance (16, 17). Conceivably, under aberrant conditions, apoptosis may generate unique “neo-antigens” for which peripheral self-tolerance has not been induced. For example, granzyme B, released during cytotoxic T lymphocyte-mediated (CTL-mediated) apoptosis of target cells during inflammatory responses, cleaves many systemic autoimmune disease-associated autoantigens at sites distinct from those of caspases (18). The possibility that variation in the apoptotic signaling pathway between cell types might

also lead to the generation of “neo-antigens” in select cell types has not been closely studied.

In PBC, as well as other inflammatory cholangiopathies, increased cholangiocyte apoptosis in the presence of activated CTLs is evident in biopsy specimens (19–21). We addressed whether PDC-E2, similar to autoantigens in several systemic autoimmune diseases, is structurally altered or becomes concentrated at the cell surface during apoptotic cell death. Immunoblot analysis of PDC-E2 using PBC patient autoantibodies indicated that PDC-E2 was not a substrate for caspase- or granzyme B-mediated cleavage and remained localized to mitochondria following apoptosis. However, there was loss of immunofluorescent staining of PDC-E2 in several noncholangiocyte cell lines (HeLa, Caco-2, Jurkat T cells, 3T3 fibroblasts, and human skin-derived fibroblasts) following apoptosis, although not in a cholangiocyte cell line, a salivary gland cell line, nor in freshly isolated intrahepatic biliary epithelial cells. Loss of PDC-E2 staining among the different cell types correlated with the expression level of Bcl-2, which has antioxidant properties and inhibits protein oxidation during cell death (22–24). Overexpression of Bcl-2 by transfection inhibited loss of PDC-E2 staining in apoptotic HeLa cells. Cholangiocytes in vivo express significantly higher levels of Bcl-2 compared with most cell types (25, 26). These results suggest that in patients with PBC, apoptotic cholangiocytes are a source of immunogenic PDC-E2 responsible for the chronic activation of autoreactive lymphocytes.

Methods

Sera and Ab's. After informed consent, sera were obtained from patients diagnosed previously with PBC, primary sclerosing cholangitis (PSC), autoimmune hepatitis (AIH), and systemic lupus erythematosus (SLE), and from normal control individuals. The diagnosis of PBC was confirmed by clinical criteria and liver biopsy in all cases. Mitotracker and mAb specific for cytochrome oxidase subunit 1 (COX-1) were purchased from Molecular Probes Inc. (Eugene, Oregon, USA). A mAb against human Bcl-2 (DAKO Inc., Glostrup, Denmark) was also obtained. All other Ab's were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, Pennsylvania, USA), unless otherwise noted.

Cell culture and apoptosis induction. HeLa (27), normal rat cholangiocytes (NRCs) (derived from cholangiocytes isolated from a normal, non-bile duct-ligated rat) (28), human skin fibroblasts, Caco-2 (29), and Jurkat T cells (30) were passaged in defined media supplemented with heat-inactivated calf serum as described previously using standard tissue culture procedures. Human salivary gland epithelial cells (HSGs) (originally from K. Shirasuna and kindly provided to us by Bruce Baum, National Institute of Dental and Cranio-facial Research, NIH, Bethesda, Maryland, USA) (31) were passaged in the same medium as the HeLa cells: DMEM supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and L-glutamine (2 mM), as well as 10% heat-inactivated calf

serum. Geneticin (200 µg/ml) and hygromycin B (100 µg/ml) were added to this medium for the passage of Bcl-2-transfected HeLa cells (generously supplied by Raymond Meyn, Jr., University of Texas, M.D. Anderson Cancer Center, Houston, Texas, USA) (originally created by Clontech Laboratories Inc., Palo Alto, California, USA) (32). The geneticin and hygromycin B were withheld 48 hours prior to each experiment. Freshly isolated rat intrahepatic bile duct epithelial cells (IBDECs) were prepared from normal rats by immunomagnetic cell separation as described previously (33) and incubated in defined media supplemented with heat-inactivated calf serum until adherent, approximately 12 hours after isolation. Once the cells were adherent, apoptosis was induced.

Apoptosis was induced by irradiation of cells with ultraviolet B light (UV-B) (1,650 J/m² for HeLa and Jurkat T cells; 2,200 J/m² for all other cells) as described previously (12). Fresh media was then added to the cells before incubation at 37°C in a humidified 5% CO₂ incubator: 6 hours for Jurkat T cells, 8 hours for HeLa, and 16 hours for the other cell types. The dose of UV-B and the length of time each cell type was kept in the incubator after irradiation was adjusted for each cell type in order to achieve equivalent amounts of apoptosis as assessed by morphologic (cytoplasmic blebbing and chromatin condensation) and biochemical (caspase cleavage of poly-ADP ribose polymerase [PARP]) criteria. For each cell type, greater than 90% cleavage of PARP was obtained after induction of apoptosis. In some experiments, buthionine sulfoximine (BSO) (100 µM) was added to the media 24 hours before UV-B irradiation, and fresh BSO-containing media was added to the cells after irradiation. Total glutathione levels were measured using a modification of the method originally described by Griffith (34). Apoptosis was alternatively induced by incubation of HeLa cells (6 hours), Caco-2 cells (16 hours), and NRCs (16 hours) in staurosporine-containing media (0.5 µM, 2.5 µM, and 2.5 µM, respectively). Serum withdrawal and granule contents purified from cytotoxic T cells were also used to induce apoptosis of HeLa cells and NRCs as described previously (18). For HeLa cells and Jurkat T cells, ligation of cell surface Fas by incubation with mAb CH11 (2 µg/ml; Kamiya Inc., Seattle, Washington, USA) for 16 hours or 6 hours, respectively, was also employed to induce apoptosis (30).

Western blot analysis of PDC-E2. Cell lysates were routinely prepared as described previously (13) in the presence of a reducing agent, DTT (5 mM). After the addition of sample buffer and boiling, samples were electrophoresed on 10% SDS-polyacrylamide gels. Protein (80 µg) was loaded in each lane. Proteins were then transferred to nitrocellulose and immunoblotted with PBC patient sera (1:2500) or SLE patient sera specific for PARP (1:10,000), NuMA (1:10,000), or U1 70K (1:10,000), three autoantigens known to be cleaved by caspases, followed by an horseradish peroxidase-conjugated (HRP-conjugated) goat anti-human IgG secondary Ab as described (13). Most of the PBC patient sera recognized PDC-E2 (human, 74 kDa; rat 66 kDa), as well as other protein bands. Two of

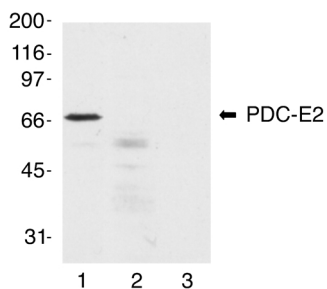


Figure 1

PBC patient sera immunoblot PDC-E2. To identify PBC sera monospecific for PDC-E2 (66 kDa), reduced NRC lysate (80 μ g per gel lane) was immunoblotted with PBC patient sera (diluted 1:2500). Two of twenty-five patient sera examined detected only a 66-kDa band (lane 1), and preincubation of these sera with purified porcine PDC (lane 2) blocked detection of this protein band. No protein band was detected when blotting only with the secondary Ab (lane 3). Molecular size markers ($M_r \times 10^{-3}$) are shown on the left. Blotting by each monospecific serum was examined three times with identical results. A representative blot is shown.

the twenty-five patient sera tested were monospecific for PDC-E2 in lysates of NRCs (Figure 1, lane 1). The identity of the lone protein band as PDC-E2 was confirmed as follows. Preincubation of the sera for 1 hour with purified porcine PDC (10 μ g/ml; Sigma Chemical Co., St. Louis, Missouri, USA) inhibited detection of the 66-kDa immunoblotted band (lane 2). No band was detected when blotting with the secondary Ab only (lane 3).

To compare PBC patient autoantibody recognition of oxidized versus reduced PDC-E2, glutathione disulfide (GSSG) (10 mM) or 5 mM DTT (5 mM), respectively, were added to lysates after the addition of SDS-containing sample buffer and boiling of the lysates. The treated lysates were then subjected to PAGE, and proteins were transferred to nitrocellulose for immunoblotting with PBC patient sera. Titrations (1,000-fold to 256,000-fold) were done for five different PBC patient sera. The subcellular localization of PDC-E2 in control versus apoptotic cells was compared by immunoblotting lysates of subcellular fractions prepared from control and apoptotic cells with PBC patient sera as described previously (35).

Immunofluorescent staining of PDC-E2. For immunofluorescent staining, cells grown on number 1 glass coverslips were washed twice in ice-cold PBS without calcium or magnesium, fixed in 4% paraformaldehyde (5 minutes at 4°C), and permeabilized in acetone (20 seconds at 4°C). Cells were stained by sequential 20-minute incubations at 4°C with monospecific patient sera (diluted 1:400) and FITC-conjugated goat anti-human IgG Ab (1:100). In some cases, cells were double-labeled by additional sequential incubations with normal goat serum (1:100) to block cross-reactivity, followed by a mouse mAb specific for COX-1 (5 μ g/ml) and a Texas red-conjugated goat anti-mouse IgG secondary Ab (1:50). Alternatively, mitotracker was used to label mitochondria. Cells were stained with 4', 6'-diamidino-2-phenylindole (DAPI; Molecular Probes Inc.) to identify

apoptotic cells by chromatin condensation and nuclear fragmentation and stained with propidium iodide (Molecular Probes Inc.) to detect cytoplasmic membrane blebbing as described previously (14). Coverslips were then mounted onto glass microscope slides (Fisher Scientific Co., Pittsburgh, Pennsylvania, USA) and confocal microscopy was performed on a scanning confocal microscope system (LSM 410; Carl Zeiss Inc., Thornwood, New York, USA). Experiments were repeated using both PDC-E2 monospecific PBC patient sera.

Immunoprecipitation of PDC-E2. Immunoprecipitation of proteins labeled with [³⁵S]-methionine from HeLa cell lysates using patient antisera was performed as previously described (18). Immunoprecipitation was repeated separately using sera from five different patients with PBC as well as from a healthy individual. Each immunoprecipitate was divided in half and treated with either GSSG (10 mM) or DTT (5 mM) before routine SDS-PAGE and autoradiograph detection of immunoprecipitated, [³⁵S]-methionine-labeled proteins.

Results

PDC-E2 is not cleaved during apoptosis. Apoptosis was induced in NRCs using UV-B irradiation. PARP, an autoantigen in SLE, is known to be cleaved by caspases during apoptosis. In reduced lysates of control and apoptotic NRCs, autoantigens were immunoblotted using PBC and SLE patient sera. The levels of intact SLE autoantigens were reduced in the apoptotic versus con-

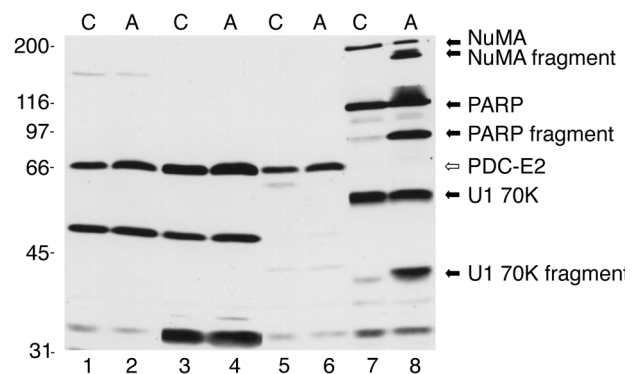


Figure 2

PDC-E2 is not cleaved during apoptosis. Reduced NRC lysates from control cells (C) and apoptotic (A) cells were immunoblotted with three different PBC patient sera (lanes 1–6) and SLE patient sera monospecific for NuMA (lanes 7 and 8, top panel), PARP (lanes 7 and 8, middle panel), and U1 70K (lanes 7 and 8, bottom panel). Apoptosis was induced by UV-B irradiation. Equal amounts of protein were electrophoresed in each gel lane. Neither loss of intact PDC-E2 (66 kDa) nor any PDC-E2 cleavage fragments were seen in the apoptotic lysates (lanes 2, 4, and 6) compared with control lysates (lanes 1, 3, and 5, respectively). Likewise, no cleavage of any other PBC autoantigen was detected. Blotting of NuMA, PARP, and U1 70K in the apoptotic lysate showed generation of their expected caspase cleavage fragments (lane 8), confirming induction of apoptosis. Molecular size markers ($M_r \times 10^{-3}$) are shown on the left. Blotting by each serum was examined at least three times with identical results. A representative blot is shown.

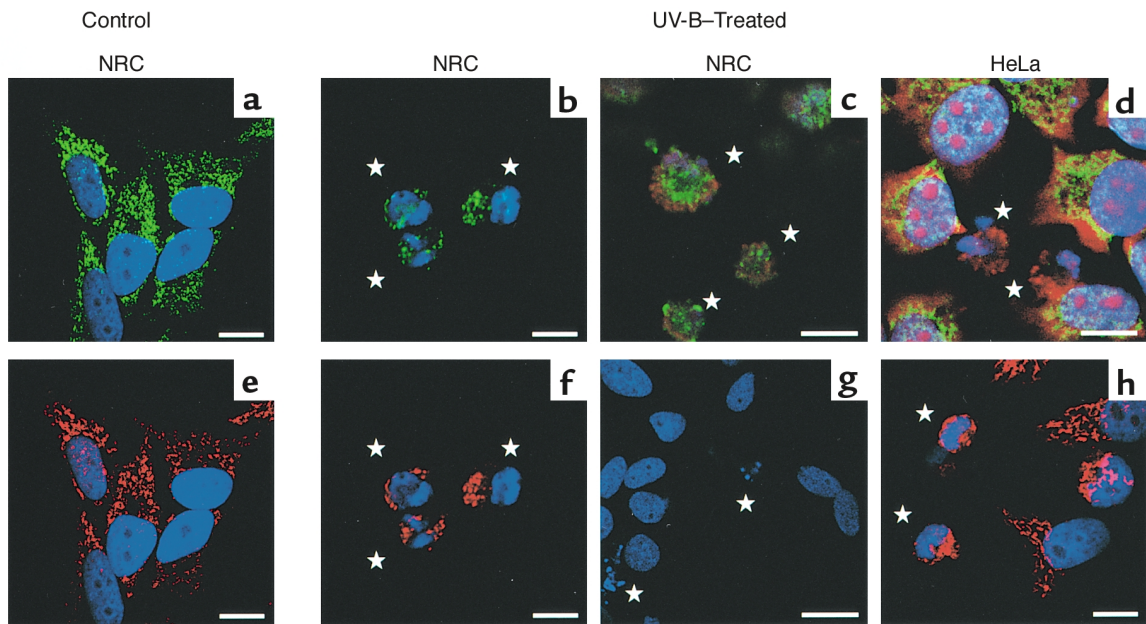


Figure 3

PDC-E2 staining by PBC patient sera in apoptotic cells does not localize to cell membrane blebs or apoptotic bodies. Both control cells and cells treated with UV-B to induce apoptosis were examined by confocal, immunofluorescence microscopy. Cells were stained with DAPI (blue) (a-h) to distinguish apoptotic cells (cells labeled with stars) with characteristic condensed, fragmented chromatin from nonapoptotic cells (unlabeled cells). Control NRC were costained with PBC patient serum monospecific for PDC-E2 (green) (a) and a mAb specific for COX-1 (red) (e). Immunoreactivity against PDC-E2 and COX-1 colocalized in mitochondria. In apoptotic NRCs, immunoreactivity against PDC-E2 (green) (b) remained perinuclear and colocalized with COX-1 (red) (f). Preincubation of PBC patient sera with purified PDC blocked staining of PDC-E2 (green) in both nonapoptotic and apoptotic NRCs (g). Cells were stained with PI (red) to distinguish cell membrane blebs or apoptotic bodies in apoptotic cells (c and d). Staining of PDC-E2 (green) in apoptotic NRCs did not localize to cell membrane blebs or apoptotic bodies (c). In contrast, immunoreactivity against PDC-E2 (green) was not detected in apoptotic HeLa cells (d), although immunoreactivity against COX-1 (red) was detected (h). Each experiment was repeated twice with each of the monospecific PBC patient sera with identical results. Bar, 20 μ m. Representative images are shown.

trol lysate (Figure 2, lanes 8 and 7, respectively) concomitant with the appearance of the expected caspase cleavage fragment of each SLE autoantigen, confirming induction of apoptosis. In contrast, no cleavage of PDC-E2 or any other PBC autoantigen was observed in the apoptotic NRC lysate (Figure 2, lanes 2, 4, and 6). Densitometric analysis of the PBC autoantigen bands showed no significant difference in intensity. Similarly, no loss of intact PDC-E2 was seen by using serum withdrawal, staurosporine, or CTL granule contents to induce NRC apoptosis (data not shown). Apoptosis was also induced in HSG, HeLa, and Jurkat T cells by UV-B irradiation as well as other apoptotic stimuli as noted in Methods. In all cases, no loss of intact PDC-E2 was observed in apoptotic lysates by immunoblotting (data not shown).

Recognition of PDC-E2 by PBC patient autoantibodies after apoptosis varies in a cell type-specific manner. The location of PDC-E2 in control and apoptotic cells was compared by indirect immunofluorescent staining. NRCs were costained with PBC patient sera monospecific for PDC-E2 and a mAb specific for COX-1, also a mitochondrial inner membrane protein. PDC-E2 stained in a punctate, perinuclear pattern (Figure 3a) typical of mitochondrial staining, which was similar to that of COX-1 (Figure 3e). PDC-E2 staining also colocalized with staining by mito-

tracker red (data not shown). Using the same secondary Ab, serum from a healthy control and sera from two patients with AIH and two patients with PSC did not stain mitochondria (data not shown). No staining was seen with secondary Ab alone (data not shown).

After UV-B irradiation to induce apoptosis, staining of PDC-E2 in apoptotic NRCs (Figure 3b; apoptotic cells are labeled with a star) again colocalized with staining of COX-1 (Figure 3f) and did not colocalize with propidium iodide (PI) staining of surface membrane blebs or apoptotic bodies (Figure 3c). Preincubation of the PBC patient sera with purified porcine PDC blocked staining of PDC-E2 in both apoptotic and nonapoptotic NRCs (Figure 3g; apoptotic cells are labeled with a star). Surprisingly, PDC-E2 in apoptotic HeLa cells was undetectable by immunostaining after UV-B irradiation (Figure 3d; apoptotic cells are labeled with a star). In contrast, UV-B-irradiated, nonapoptotic HeLa cells (cells lacking chromatin condensation and membrane blebbing) stained strongly for PDC-E2 (Figure 3d; cells not marked with a star). In contrast, COX-1 staining in apoptotic HeLa (Figure 3h) was readily detectable and similar to that in apoptotic NRCs (Figure 3f).

To better understand this difference in PDC-E2 staining in apoptotic HeLa cells compared with apoptotic

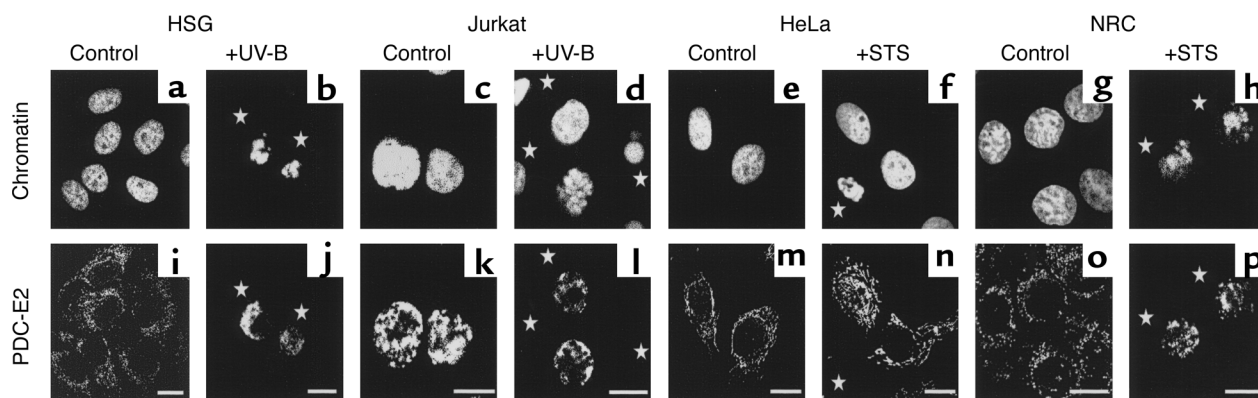


Figure 4

Persistence of PDC-E2 staining by PBC patient sera in apoptotic cells is cell-type dependent and independent of the stimulus used to induce apoptosis. Both control cells and cells treated with UV-B or staurosporine (STS) to induce apoptosis were examined by confocal, immunofluorescence microscopy. Cells were stained with DAPI (a–h) to distinguish apoptotic cells (cells labeled with stars) from nonapoptotic cells (unlabeled cells). After UV-B treatment, PBC patient sera staining of PDC-E2 was detected in apoptotic HSGs (j), but not in apoptotic Jurkat T cells (l). When inducing apoptosis with STS, PDC-E2 immunoreactivity again was undetectable in apoptotic HeLa (n), while persisting in apoptotic NRCs (p), and was undetectable in apoptotic HeLa (n). Bars, 20 μ m. All cell types were stained independently with two different PDC-E2 monospecific sera at least three times with similar results each time. Representative images are shown.

NRCs, we examined both additional cell types and other apoptotic stimuli. After UV-B treatment, there was strong staining of PDC-E2 in apoptotic HSGs (Figure 4j) as in NRCs, while there was loss of PDC-E2 staining in apoptotic Jurkat T cells (Figure 4l) as in HeLa cells. HSGs were studied since salivary gland damage in PBC is quite common. Loss of PDC-E2 staining in apoptotic cells was also observed after staurosporine treatment of HeLa cells (Figure 4n; apoptotic cells are labeled with stars), Caco-2 cells (data not shown), and Jurkat T cells (data not shown). In contrast, staurosporine treatment of neither NRCs (Figure 4p) nor HSGs (data not shown) led to a loss of PDC-E2 staining in apoptotic NRCs or HSGs. Likewise, CTL granule treatment caused a loss of PDC-E2 staining in apoptotic HeLa cells, but not in apoptotic NRCs (data not shown). For each stimulus, persistence of PDC-E2 staining in apoptotic cells was dependent on the cell type undergoing apoptosis.

Loss of autoantibody recognition of PDC-E2 after apoptosis is due to glutathiolation of its sulfhydryl group(s). Possible explanations for the loss of PDC-E2 staining in certain cell types after apoptosis include the following: (a) leakage of PDC-E2 out of the mitochondria, (b) degradation of PDC-E2, and (c) loss only of the PDC-E2 epitope(s) recognized by PBC patient autoantibodies. To detect leak-

age of PDC-E2 into the cytosol after UV-B-induced apoptosis, subcellular fractions were prepared from control and apoptotic HeLa cells. The high degree of PARP cleavage detected in the nuclear fraction of UV-B-treated cells (Figure 5, lane 2) confirmed that apoptosis was induced in a high percentage of the cells. Little PDC-E2 was detected in the lysate of the apoptotic cytosol fraction (Figure 5, lane 4). Additionally, there was equivalent immunoblotting of PDC-E2 in the apoptotic mitochondrial lysate as compared with the control mitochondrial lysate (Figure 5, lanes 6 and 5, respectively). Since autoantibody recognition of PDC-E2 by immunoblotting (Figure 2) was equivalent in lysates of control and apoptotic cells, degradation of PDC-E2 is an unlikely explanation. Loss of only the PDC-E2 epitope recognized by PBC patient autoantibodies cannot be similarly ruled out by the immunoblotting results since loss of the epitope may be transient and dependent on the local mitochondrial environment.

Previous studies of PDC purified from tissue specimens have shown that the PDC-E2 epitope recognized by PBC patient autoantibodies is sensitive to the sulfhydryl redox potential of the preparation (36). The intensity of PDC-E2 immunoblotting by PBC patient antisera correlated with the concentration of the

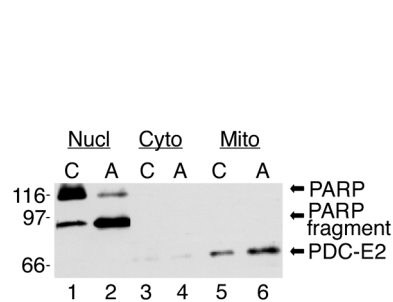


Figure 5

PDC-E2 does not leak out of mitochondria during apoptosis. Reduced lysates of subcellular fractions of control (C) and UV-B-irradiated, apoptotic (A) HeLa cells were immunoblotted with SLE and PBC patient sera monospecific for PARP (lanes 1 and 2) and PDC-E2 (lanes 3–6), respectively. Intact PARP predominated in the control nuclear (Nucl) fraction (lane 1), while its caspase cleavage fragment was the predominant form detected in the apoptotic nuclear fraction (lane 2), confirming induction of a high degree of caspase activity in the UV-B-treated cells. PDC-E2 was strongly detected in both control and apoptotic mitochondrial (Mito) fractions (lanes 5 and 6), but not in either cytosolic (Cyto) fraction (lanes 3 and 4). Molecular size markers ($M_r \times 10^{-3}$) are shown on the left. The experiment was repeated twice with identical results. A representative blot is shown.

Table 1

Cellular glutathione levels in the presence and absence of BSO

Cell type	-BSO	+BSO	+UV-B
NRCs	19 ± 5	0.1 ± 2	NA
HSGs	17 ± 4	0.6 ± 1	NA
HeLa	9.5 ± 2	0.2 ± 1	0.3 ± 1
HeLa-Bcl-2	15 ± 6	NA	12 ± 4

Cells were cultured in defined media with or without BSO (100 μ M) for 24 hours before preparing lysates for the determination of total glutathione and protein levels. Similar measurements were made for untreated cells after induction of apoptosis by UV-B irradiation. Each experiment was performed at least three times. Results are expressed as nanomoles of glutathione per milligram of protein \pm SEM.

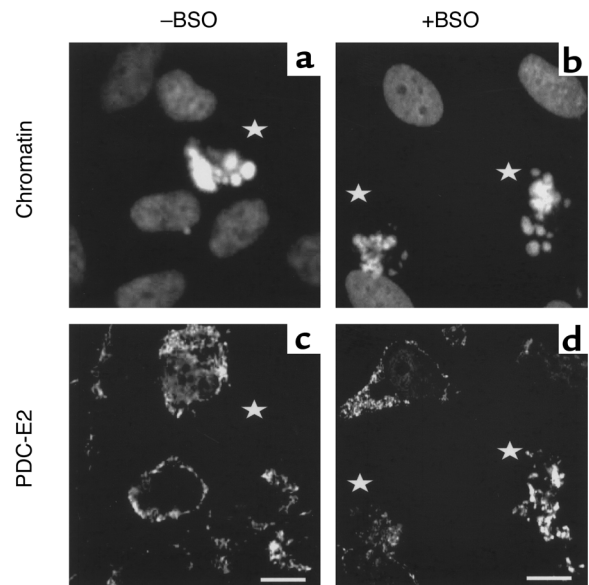
sulfhydryl reducing agent in the sample buffer added to purified PDC before SDS-PAGE, suggesting that PBC patient autoantibodies preferentially recognize PDC-E2 with reduced sulfhydryl groups. Coincidentally, the cellular sulfhydryl redox potential during apoptosis has been shown to decrease during apoptosis in some cell types (37) due to increased reactive oxygen species (ROS) production, particularly within the mitochondria.

We have shown previously that treatment of cholangiocytes with BSO, a glutathione synthetase inhibitor, depletes cholangiocytes of glutathione (38), increasing their sensitivity to the effects of ROS. To determine if the cell type-specific difference in PDC-E2 staining after apoptosis was due to differences in the cellular sulfhydryl redox potential and subsequent ROS production during apoptosis, NRCs were pretreated with BSO for 24 hours before inducing apoptosis. HSG and HeLa cells were similarly treated. Pretreatment of each cell type with BSO reduced total glutathione levels greater than 90% compared with untreated cells (Table 1) and did not by itself induce apoptosis. There was no difference in PDC-E2 staining of apoptotic NRCs and HSG cells with or without BSO treatment before UV-B irradiation (data not shown). Unexpectedly, staining of PDC-E2 persisted in apoptotic HeLa cells after UV-B irradiation of cells pretreated with BSO (Figure 6d). As before, in the absence of pretreatment with BSO, PDC-E2 staining was undetectable in apoptotic HeLa cells (Figure 6c; apoptotic cells are labeled with a star). Preincubation of the PBC patient sera with purified PDC inhibited staining of PDC-E2 in apoptotic HeLa cells pretreated with BSO (data not shown). Depletion of total cellular glutathione with BSO prevented the loss of PDC-E2 staining after HeLa cell apoptosis.

This result suggested a role for glutathione (GSH) in the oxidation of PDC-E2 sulfhydryl groups leading to the loss of PDC-E2 staining in apoptotic HeLa cells. Increased concentrations of protein-S-S-G conjugates in apoptotic cells have been reported (24). To determine if oxidized glutathione (GSSG) directly oxidizes PDC-E2 sulfhydryl groups to cause loss of the PDC-E2 epitope recognized by PBC patient autoantibodies, detergent lysates of apoptotic NRCs were treated with DTT (5mM) and SDS (2%) and then boiled to inactivate lysate pro-

teins before the addition of GSSG (10mM) and subjected to PAGE. After transfer of proteins to nitrocellulose, immunoblotting of PDC-E2 by PBC patient autoantibodies was absent in lysate to which GSSG was added (Figure 7a, lane 2). In the absence of GSSG (lane 1) or in the presence of excess DTT (25 mM) relative to GSSG (10 mM) (lane 3), PDC-E2 was strongly detected. Glutathiolation of PDC-E2 in apoptotic lysate reversibly inhibited its recognition by PBC patient autoantibodies.

In titration experiments using serum from five different patients with PBC, the difference in autoantibody recognition of PDC-E2 in GSSG-treated versus DTT-treated lysates of apoptotic NRCs ranged from 100- to 1,000-fold (Figure 7b and data not shown) based on the calculated autoantibody dilution at which the OD of the PDC-E2 band would equal 1.5 (the midpoint of the OD scale). That poor autoantibody recognition of PDC-E2 in GSSG-treated lysates was not due to inefficient transfer of glutathiolated PDC-E2 to nitrocellulose was demonstrated as follows. PDC-E2 was oxidized (GSSG treated) or reduced (DTT treated) after its immunoprecipitation from lysates of [³⁵S]-methionine-labeled HeLa cells with antisera from patients with PBC. After SDS-PAGE, both oxidized and reduced [³⁵S]-methionine labeled PDC-E2 transferred to nitrocellulose in equal amounts as assessed by autoradiography (Figure 7c).

**Figure 6**

Depletion of cellular glutathione by BSO treatment before UV-B irradiation prevents the loss of the PDC-E2 staining in apoptotic HeLa cells. UV-B-irradiated cells were stained with DAPI (a and b) to differentiate nonapoptotic from apoptotic cells and with monospecific PBC patient serum against PDC-E2 (c and d). Apoptotic cells are labeled with stars. Staining was analyzed by confocal, immunofluorescence microscopy. In the absence of pretreatment with BSO, staining of PDC-E2 was again not detected in apoptotic HeLa cells after UV-B irradiation (c). However, staining of PDC-E2 persisted in apoptotic HeLa cells pretreated with BSO before UV-B irradiation (d). Bars, 20 μ m. Each experiment was repeated at least three times with identical results. Representative images are shown.

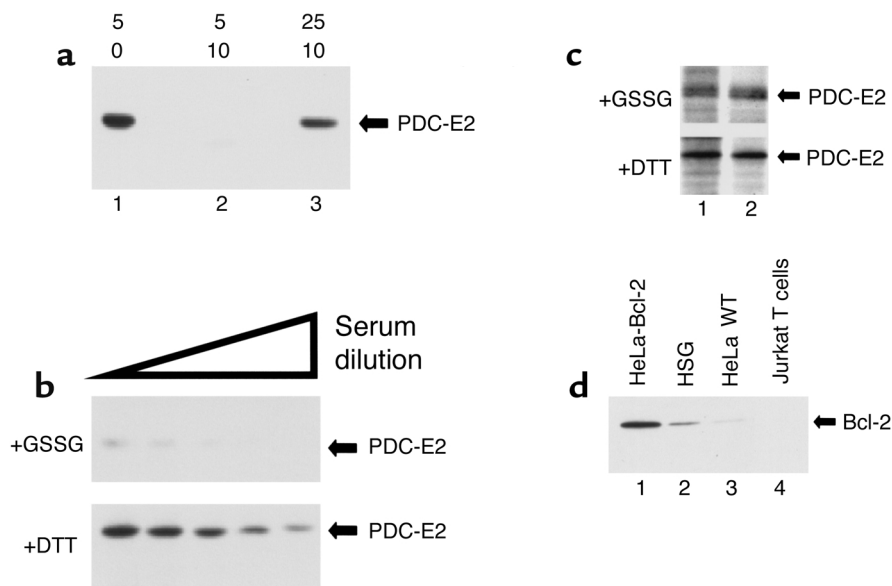


Figure 7

Direct oxidation of PDC-E2 by oxidized glutathione abrogates recognition of PDC-E2 by PBC patient sera. (a) Apoptotic NRC lysate was treated with SDS, boiled, and then sequentially treated with 5 mM DTT, 10 mM GSSG, and 25 mM DTT. Strong blotting of PDC-E2 was detected in lysate treated with 5 mM DTT (lane 1). Blotting of PDC-E2 was absent after addition of GSSG to the DTT-treated lysate (lane 2), but subsequent treatment with additional DTT restored detection of PDC-E2 (lane 3). (b) The difference between recognition of oxidized versus reduced PDC-E2 by PBC patient autoantibodies was quantified by immunoblotting lysate treated with 10 mM GSSG vs. 5 mM DTT. A representative blot is shown. (c) The oxidative state of PDC-E2 does not significantly affect its transfer to nitrocellulose. PDC-E2 was immunoprecipitated using PBC patient sera from lysate of HeLa cells incubated with [³⁵S]-methionine to label proteins. Half the immunoprecipitate was treated with DTT and the other half with GSSG before SDS-PAGE was performed. The autoradiogram performed after transfer to nitrocellulose showed that similar amounts of both reduced and oxidized PDC-E2 were transferred. Results using two different PBC patient sera are shown (lanes 1 and 2). (d) The expression level of Bcl-2 correlates with persistence of PDC-E2 staining following apoptosis. Eighty micrograms of lysate protein was loaded per lane and immunoblotted with an mAb specific for human Bcl-2. High levels of Bcl-2 were only detected in the Bcl-2-transfected HeLa (lane 1) and HSG cell lysates (lane 2).

High-level Bcl-2 expression preserves the PDC-E2 epitope recognized by PBC patient autoantibodies in apoptotic cells. The formation of protein-S-S-G conjugates during apoptosis is regulated by the expression level of Bcl-2, an antiapoptotic protein with antioxidant properties (24). Human cholangiocytes (25, 26) and NRCs (39) are unusual in that they constitutively express high levels of Bcl-2. High levels of Bcl-2 were detected in HSG cells as well (Figure 7d, lane 2), but not in HeLa or Jurkat T cells (Figure 7d, lanes 3 and 4, respectively). The expression level of Bcl-2 in each cell type correlated with persistence of PDC-E2 staining after apoptosis. To directly address whether the level of Bcl-2 expression affects glutathiolation of PDC-E2 in apoptotic cells, HeLa cells expressing high levels of Bcl-2 (Figure 7d, lane 1) after transfection with a Bcl-2 cDNA were studied.

UV-B-irradiated, Bcl-2-transfected HeLa cells were stained with PBC patient sera monospecific for PDC-E2. To achieve an equivalent extent of apoptosis, the incubation time following UV-B irradiation was doubled in the Bcl-2-transfected HeLa cells compared with wild-type (WT) HeLa cells. Intense punctate, perinuclear staining was detected in apoptotic, Bcl-2-transfected HeLa cells (Figure 8h; apoptotic cells are labeled with a star), unlike apoptotic, WT HeLa cells (Figure

3d). The PDC-E2 epitope(s) recognized by PBC patient autoantibodies persisted after apoptosis of Bcl-2-transfected HeLa cells, but not WT HeLa cells. Consistent with this finding, glutathione levels are 50% higher in nonirradiated Bcl-2-transfected HeLa cells than WT HeLa cells and only decrease dramatically in the WT HeLa cells, not in the Bcl-2-transfected HeLa cells, after UV-B irradiation (Table 1). To strengthen the correlation between cellular Bcl-2 levels and preservation of the PDC-E2 epitope recognized by PBC patient autoantibodies after apoptosis, primary human skin fibroblasts, known to express low levels of Bcl-2 (40), were also examined. In apoptotic primary fibroblasts, PBC patient autoantibody staining of PDC-E2 was absent after UV-B irradiation (Figure 8j), as observed in cell lines expressing relatively low levels of Bcl-2. In both cell lines and primary cells, persistence of the PDC-E2 epitope recognized by PBC patient autoantibodies in apoptotic cells correlated with the expression level of Bcl-2 in that cell type. Additionally, staining of PDC-E2 persisted in freshly isolated, rat IBDECs after UV-B induced apoptosis (Figure 8i) as observed with NRCs, suggesting that the studies of NRC apoptosis pertain to apoptosis of native cholangiocytes lining the intrahepatic biliary tree.

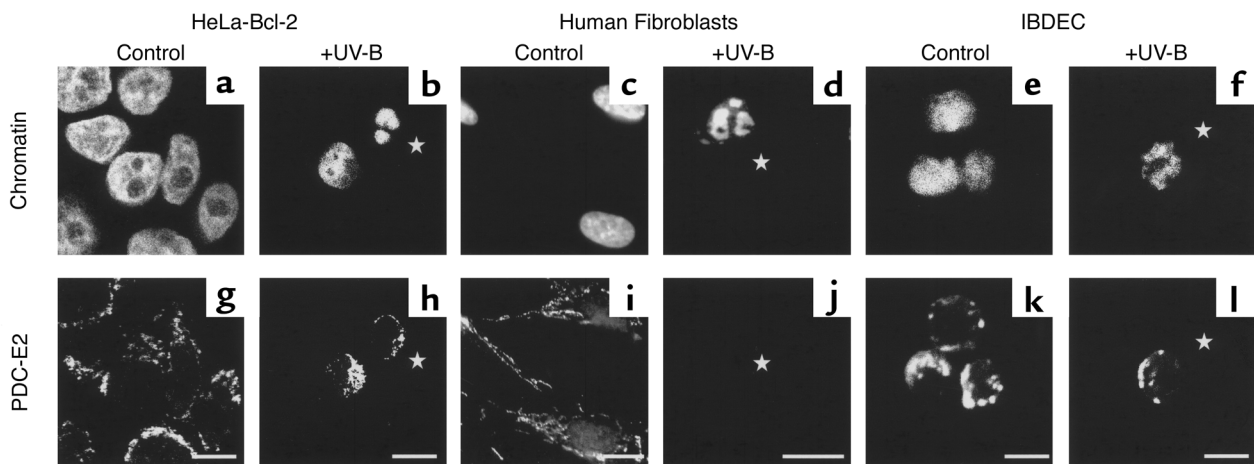


Figure 8

Oxidation of PDC-E2 during apoptosis is Bcl-2 dependent. Control and UV-B-irradiated cells were stained with DAPI (a-f) to differentiate non-apoptotic from apoptotic cells and with monospecific PBC patient serum against PDC-E2 (g-l). Apoptotic cells are labeled with stars. Staining was analyzed by confocal, immunofluorescence microscopy. Staining of PDC-E2 was present in apoptotic, Bcl-2-transfected HeLa cells (h) and apoptotic, freshly isolated IBDECs (j), though not in apoptotic, primary fibroblasts (l). Bars, 20 μ m. Representative images are shown.

Discussion

The selective modification of autoantigens during apoptosis has led to the suggestion that apoptotic cells may be a source of immunogen in patients with autoimmune disease. A high percentage of autoantigens are cleaved by caspases or otherwise altered during apoptosis and become concentrated in surface membrane blebs or apoptotic bodies. These studies show that PDC-E2 is not cleaved by caspases during apoptosis, nor does it become concentrated at the cell surface. However, in most cell types, PDC-E2 did undergo a structural alteration during apoptosis, as detected by the loss of the PDC-E2 epitope(s) recognized by PBC patient autoantibodies. A number of different apoptosis-inducing stimuli had the same effect on recognition of PDC-E2. Loss of recognition of this antigenic epitope(s) was apparently due to the covalent modification of a PDC-E2 sulfhydryl group by glutathione to form a mixed disulfide (PDC-E2-S-SG). These results indicate that the reduced form of PDC-E2, not glutathiolated PDC-E2, is responsible for the activation of autoreactive B cells specific for PDC-E2 in patients with PBC. Significantly, cholangiocytes and salivary gland epithelial cells, the cell types most frequently affected in patients with PBC, retain the PDC-E2 epitope(s) recognized by PBC patient autoantibodies after apoptosis. Thus, a lack of PDC-E2 modification after apoptosis appears to be unusual and preserves its antigenic epitope.

In each cell type, autoantibody recognition of PDC-E2 after apoptosis correlated with the expression level of Bcl-2. Bcl-2 expression has been shown to inhibit oxidative protein damage during cell death (22, 23). Under the conditions used to induce apoptosis, expression of Bcl-2 retarded, but did not prevent, apoptosis. Additionally, overexpression of Bcl-2 by transfection in HeLa cells preserved recognition of PDC-E2 after apoptosis, in contrast to WT HeLa cells. Thus, Bcl-2 appears to inhibit protein glutathiolation during apoptosis. The apparent mecha-

nism by which Bcl-2 inhibits oxidative damage during apoptosis varies among cell types and remains unclear in some cases. In Bcl-2-transfected HeLa, Bcl-2 may act by increasing basal glutathione levels (41). Intrahepatic cholangiocytes express high levels of Bcl-2, unlike most other cell types in vivo (25). Thus, B cell exposure to reduced PDC-E2 from damaged and dying cells is likely minimal. Therefore, we postulate that unusually high concentrations of the reduced form of PDC-E2 following intrahepatic cholangiocyte damage and death are in part responsible for the activation of autoreactive B cells in patients with PBC. Additional factors clearly regulate induction of high titer IgG anti-PDC-E2 Ab production since other conditions involving increased cholangiocyte cell death are not associated with the production of autoantibodies specific for PDC-E2.

The bile duct damage in PBC is considered more likely to be mediated by activated T cells than by autoantibodies (42, 43). T cell activation requires the MHC-restricted presentation of peptides by antigen-presenting cells (APCs), such as macrophages and dendritic cells. Previous studies of two non-PBC autoantigens, insulin (44) and beta2-glycoprotein I (45) have shown that APC processing of only the sulfhydryl-reduced forms of these autoantigens leads to activation of autoreactive T cells. While PDC-E2 is endogenously expressed by APCs, APCs have been shown to present different peptides of the same antigen, depending on whether the antigen is expressed endogenously or engulfed from an exogenous source, such as apoptotic cells (46). Determining whether or not the oxidative state of PDC-E2 sulfhydryl groups also affects activation of autoreactive T cells in patients with PBC may further aid in understanding the pathogenesis of this disease.

The suggestion that apoptotic cholangiocytes are a source of immunogen in PBC implies that the activation of autoreactive lymphocytes in PBC is an epiphenome-

non following bile duct damage. Yet, autoantibodies are typically detected in PBC patients with early-stage disease when there is little or no evidence of bile duct damage. Interestingly, the aberrant expression of MHC class II molecules on the basolateral surface of intrahepatic cholangiocytes in patients with PBC indicates that the cholangiocytes, themselves, act as APCs (47). Presentation by healthy cholangiocytes of immunogenic PDC-E2 peptides derived from reduced PDC-E2 in engulfed apoptotic cholangiocytes could lead to further cholangiocyte destruction by autoreactive T cells. Thus, a self-sustaining cycle of cholangiocyte destruction might develop after an initial, unidentified triggering event in susceptible individuals. Whether or not healthy cholangiocytes engulf apoptotic cells, as shown for hepatocytes (48), has not been reported. Previous immunohistochemical studies have detected aberrant staining by PDC-E2-specific Ab's of the apical cell surface and subapical region of PBC patient cholangiocytes (49). Engulfed apoptotic debris is a potential source of this aberrant staining.

Previous studies have directly demonstrated the glutathiolation of a limited subset of soluble cellular proteins in response to increased ROS production after TNF- α stimulation (50). Similar techniques may be useful to confirm directly that PDC-E2 as well as other PBC autoantigens are glutathiolated during apoptosis in most cell types. Sulfhydryl groups are often critical residues within the functional domains of proteins. Thus, protein glutathiolation may play a role in cell signaling by regulating the function of proteins with oxidant-sensitive sulfhydryl groups. With regard to apoptosis signaling, Costantini et al. (51) recently demonstrated that oxidation of a critical sulfhydryl group of the mitochondrial adenine nucleotide transporter (ANT) is sufficient to facilitate mitochondrial membrane permeability transition, which enhances the release from the mitochondria of cytochrome *c* (52, 53) and apoptosis-inducing factor (54). The release of these factors results in the activation of effector caspases, changes in nuclear morphology, and increased ROS production. Coincidentally, the liver-specific isoform of ANT has been identified previously as a PBC patient autoantigen (55).

Our studies do not indicate which PDC-E2 sulfhydryl group(s) is potentially oxidized during apoptosis. mAb's directed at different epitopes of PDC-E2 may be useful in this regard, as well as to confirm our cell fractionation results (see Figure 5) showing that PDC-E2 levels remain constant in the mitochondria of apoptotic cells even when the epitope recognized by PBC patient autoantibodies is lost. The major PDC-E2 epitope recognized by PBC patient autoantibodies maps to the inner lipoyl domain of PDC-E2, which contains sulfhydryl groups involved in enzyme function (56, 57). Critical sulfhydryl groups are also present within the antigenic sites of several other PBC autoantigens (58, 59). Additionally, PBC patient autoantibodies inhibit PDC activity *in vitro* (60). Therefore, glutathiolation of PDC-E2 would likely inhibit its enzymatic activity leading to decreased production of acetyl CoA and citric

acid cycle activity. Since loss of PDC-E2 staining was not observed in apoptotic HeLa cells without concomitant changes in nuclear morphology, glutathiolation of PDC-E2 likely followed cytochrome *c* release and did not contribute to the induction of apoptosis. Further studies are needed to determine if rapid glutathiolation of PDC-E2 after a toxic stimulus might favor necrotic rather than apoptotic cell death.

PDC-E2 is the first major autoantigen for which autoantibody recognition of its antigenic epitope has been shown to be lost after apoptosis due to oxidation of a sulfhydryl group(s). Autoantibody recognition of many other autoantigens, such as GAD65 (61), a major autoantigen in patients with type I diabetes mellitus, and fibrillarin (62), associated with scleroderma, is also dependent on the oxidative state of the autoantigen. In type I diabetes mellitus, pancreatic β islet cell apoptosis is involved in the pathogenesis of the disease (63–65). Whether or not GAD65 is oxidized during apoptosis of pancreatic β islet cells or any other cell type has not been determined. Our findings with PDC-E2 suggest that autoantigen oxidation during apoptosis may be cell-type specific.

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