Mechanisms Of Mitochondrial Damage In Keratinocytes By Pemphigus Vulgaris Antibodies*

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CAPSULE

Background:
Previous studies suggested that mitochondrial antibodies contribute to pemphigus vulgaris (PV).

Results:
PV sera elicited mitochondrial damage, and mitochondria-protecting drugs exhibited protective effect in cell culture and mouse skin.

Conclusion:
PV antibodies altered O2 respiration, disrupted electron transfer chain and increased reactive oxygen species.

Significance:
Results provide the mechanism of therapeutic action and justify the use of mitochondria-protecting drugs in PV.
ABSTRACT

The development of non-hormonal treatment of pemphigus vulgaris (PV) has been hampered by a lack of clear understanding of the mechanisms leading to keratinocyte (KC) detachment and death in pemphigus. In this study, we sought to identify changes in the vital mitochondrial functions in KCs treated with the sera from PV patients and healthy donors. PV sera significantly increased proton leakage from KCs, suggesting that PV IgGs increase production of reactive oxygen species (ROS). Indeed, measurement of intracellular ROS production showed a drastic increase of cell staining in response to treatment by PV sera, which was confirmed by FACS analysis. Exposure of KCs to PV sera also caused dramatic changes in the mitochondrial membrane potential detected with the JC-1 dye. These changes can trigger the mitochondria-mediated intrinsic apoptosis. While sera from different PV patients elicited unique patterns of mitochondrial damage, the mitochondria-protecting drugs nicotinamide, minocycline and cyclosporine A exhibited a uniform protective effect. Their therapeutic activity was validated in the passive transfer model of PV in neonatal BALB/c mice. The highest efficacy of mitochondrial protection of the combination of these drugs found in mitochondrial assay was consistent with the ability of the same drug combination to abolish acantholysis in mouse skin. These findings provide a theoretical background for clinical reports of the efficacy of mitochondria-protecting drugs in PV patients. Pharmacological protection of mitochondria and/or compensation of an altered mitochondrial function may therefore become a novel approach to development of personalized non-hormonal therapies of patients with this potentially lethal autoimmune blistering disease.
INTRODUCTION

Pemphigus vulgaris (PV) is a life-long, IgG autoantibody-mediated blistering disease affecting oral/esophageal surfaces and/or skin. Patients develop blisters and non-healing erosions due to suprabasal split within the stratified squamous epithelium. Prior to the introduction of therapy with oral corticosteroids in the 1950s, PV had a dismal natural course with a 50% mortality rate at 2 years and 100% mortality rate by 5 years after onset of the disease. While systemic corticosteroid treatment is life saving, the high-dose and prolonged courses required for disease control are associated with significant adverse effects, including death (1,2). At present, mortality remains at a relatively high level of up to 12% (3). The optimal therapeutic strategy has not been established, the steroid-sparing drugs are associated with significant adverse effects themselves, and it is unknown which is the preferable steroid-sparing agent (4). The ultimate goal of pemphigus research is to develop an effective treatment modality that would allow patients to achieve and maintain clinical remission without the need for systemic corticosteroids.

The mechanism of detachment of keratinocytes (KCs) in PV, termed acantholysis, remains obscure and is a subject of intensive research. On the cell membrane of KCs, IgG autoantibodies produced by PV patients react with desmoglein (Dsg) 3 ± 1 and other self-antigens, elicit downstream signaling events causing cell shrinkage, detachment from neighboring KCs and rounding up (reviewed in (5)). While the pathogenic role of anti-Dsg antibodies is well-characterized (reviewed in (6)), our recent studies using proteomic technology identified novel targets of PV autoimmunity (7,8). Various anti-keratinocyte antibodies may concur to cause blistering by acting synergistically with anti-Dsg antibodies, as has been described through the "multiple hit" hypothesis (9). Our focus on mitochondrial targets of PV autoimmunity stems from classic and modern studies placing mitochondrial damage within the pathophysiological loop. The mitochondrial dysfunction in the lesional skin of PV patients had been suggested by an increase of lipid peroxidation and peroxidant-antioxidant balance, reflecting an increased production of reactive oxygen species (ROS) (10,11) and measuring oxidative stress (12), respectively, abnormal activities of the mitochondrial enzymes oxidoreductase, adenosine triphosphatase and NADH2-cytochrome c reductase (13-15) and skewed balance between oxybiotic and anoxybiotic metabolism towards the latter (15).

Studies of mitochondrial antibodies (MtAbs) in pemphigus were pioneered by Geoghegan and Jordon in 1992 (16) and further developed by our group. We became interested in MtAbs because we sought to elucidate the mechanism of intrinsic apoptosis of KCs in PV originally demonstrated by us (17) and confirmed by other workers (18,19). The direct evidence that MtAbs are critical to disease pathology, rather than a bystander phenomena in PV, was provided by the studies demonstrating that PV IgGs enter KCs and specifically bind to a number of mitochondrial proteins, which is associated with the mitochondrial damage manifested by cytochrome c release (20). Most importantly, adsorption of MtAbs abolished the ability of the IgG fraction of PV serum (PV IgG) to cause keratinocyte detachment (ie, acantholysis) and skin blistering, thus illustrating their pathophysiological significance. Using a protein microarray approach, we have recently analyzed antigen-specificities of autoantibodies of a large cohort of pemphigus patients and identified a number mitochondria-associated proteins targeted by MtAbs (8) The most common targeted for MtAbs in PV are listed in Table 1. On the other hand, there is a growing evidence that the pharmacological agents that can protect
mitochondria, such as minocycline, nicotinamide and cyclosporine A, are therapeutic in PV patients (Table 2). Thus, taken together, the existing data has suggested strongly that PV IgG binding to KCs causes mitochondrial dysfunction and oxidative stress, triggering apoptosis of KCs and acantholysis (a.k.a. apoptolysis (21)), and that correction of mitochondrial function may be therapeutic in PV.

In this study, we employed assays of mitochondrial functions to identify changes in the vital mitochondria functions, such as O2 respiration, mitochondrial membrane potential ($\Delta \Psi_m$) and intracellular production of ROS, in KCs treated with the sera from PV patients and healthy donors. The obtained results indicated that MtAbs produced by PV patients can disrupt the electron transfer chain, resulting in a loss of electrochemical gradient across the inner membrane, increase ROS production and reduce the ability of KCs to respond to stress. While individual PV patient's MtAbs elicited a unique pattern of mitochondrial damage, mitochondria-protecting drugs exhibited a uniform protective effect. Their therapeutic activity was validated in the passive transfer PV model in neonatal BALB/c mice. The obtained results explain the mechanism of therapeutic action of mitochondria-protecting drugs in PV patients and suggest novel avenues for treatment of this potentially lethal immunoblistering disease.

**MATERIALS AND METHODS**

**Test sera and cells**

We tested 6 PV patient and 6 normal serum specimens. This study was approved by the University of California Irvine Human Subjects Review Committee. The diagnosis of PV was made based on the results of comprehensive clinical and histological examinations, and immunological studies that included both direct immunofluorescence of skin biopsies and indirect immunofluorescence of the patients’ sera on various epithelial substrates. The titer of "intercellular" antibodies determined on monkey esophagus ranged from 1/320 to 1/2560. The presence of anti-Dsg 1 and Dsg 3 antibodies in each PV serum was established using the MESACUP Dsg 1 & Dsg 3 ELISA test system (MBL, Nagoya, Japan). The index values for Dsg 1 antibodies ranged from 26 to 72 and those for Dsg 3 antibodies—from 65 to 164, ie, were unequivocally positive. Patient specimens were de-identified prior to testing. As controls, we used normal human sera purchased from Bioreclamation, Inc. (Westbury, NY). The Het-1A cell line—an established clonal population of SV40-immortalized human esophageal squamous epithelial cells (ie, KCs) widely used for the studies of apoptosis (22)—was purchased from American Type Culture Collection (Manassas, VA; catalog no. CRL-2692) and propagated in the Clonetics brand bronchial cell medium without retinoic acid (Cambrex Bio Sciences, Walkersville, MD), as detailed by us elsewhere (23).

**Analysis of mitochondrial O2 respiration by extracellular flux measurement**

To measure mitochondrial function in Het-1A cells, we employed a Seahorse Bioscience XF24 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA) and followed the manufacturer’s protocol. Briefly, KCs were plated in a 0.2% gelatin coated 24-well Seahorse XF-24 assay plate at 3 x 10^4 cells/well, and grown for 16 hrs before being treated in quintuplicate with either PV patient’s or normal human sera at a final concentration of 4% for another 24 hrs in the culture media. On the day of metabolic flux analysis, cells were washed once with freshly prepared Krebs-Henseleit buffer (KHB; 111 mM NaCl, 4.7 mM KCl, 2 mM MgSO4, 1.2 mM Na2HPO4, 2.5 mM glucose and 0.5 mM carnitine; pH 7.4) and incubated in KHB buffer at 37°C in a non-CO2 incubator for 1 hr. Three baseline measurements of oxygen consumption rate (OCR) were taken before sequential injection of the following mitochondrial inhibitors and final concentration: oligomycin
(1 μg/ml), FCCP (3 μM) and rotenone (0.1 μM). Three measurements were taken after addition of each inhibitor. The OCR values were automatically calculated and recorded by the Seahorse XF-24 software. The basal respiration was calculated by averaging the three measurements of OCR before injection of inhibitors. The proton leak was calculated using OCR measurement after oligomycin injection minus OCR measurement after rotenone injection.

**Measurement of mitochondrial ΔΨm using the JC-1 dye**

The changes in ΔΨm induced by test sera were measured using a standard protocol (24). Briefly, Het-1A cells were plated in a 6-well plate at a density of 5 x 10⁴ per well, incubated for 16 hrs to allow cells to adhere to the dish surface, after which the cells were either left untreated (negative control) or exposed for 24 hrs to PV or control sera at a final concentration of 4% of the total volume per well. Next, experimental and control cells were exposed to the fluorescent cationic dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) for 25 min before observation at green and red emission wavelengths using a Zeiss AxioPlan II fluorescence microscope (Carl Zeiss, Thornwood, NY). Intact mitochondrial membrane retains the JC-1 dye and forms J-aggregate (orange-red fluorescence), while depolarization of membrane decreases the ability to retain the dye that remains as a monomer (green fluorescence). Increased green fluorescence and decreased orange-red fluorescence thus indicated loss of ΔΨm. In the absence of apoptosis, the lipophilic JC-1 dye bearing a delocalized positive charge accumulates in the mitochondrial matrix and stains the mitochondria bright red, whereas in mitochondrial apoptotic ΔΨm dissipates the cells display green fluorescence.

**Intracellular ROS measurement**

The measurement of intracellular ROS was performed in accordance to the manufacturer’s protocol. The Het-1A cells were incubated and treated as described above in the ΔΨm measurement section. Briefly, to measure intracellular ROS, the treatment media was removed, and 5 μM c-H₂DCFDA-AM (Invitrogen, Grand Island, NY) in PBS, pH 7, was added to each well and incubated for 15 min at 37°C. The cells were then photographed using a fluorescence microscope. C-H₂DCFDA-AM is hydrolyzed by intracellular esterases and oxidized to fluorescent carboxy-DCF (excitation 488 nm; emission 525 nm).

**FACS measurements of ΔΨm and ROS**

For the FACS analysis, the experimental and control Het-1A cells were incubated at 37°C with either 5 μM of JC-1 dye for 60 min (ΔΨm measurement) or 5 μM of c-H₂DCFDA-AM for 15 min (ROS measurement), washed three times with PBS, trypsinized and resuspended in PBS. The fluorescence was measured with BD LSR II Flow Cytometer (excitation at 488 nm and emission at 576 nm), and the data were analyzed using the BD FACSDiVa software.

**Morphometric assay of acantholysis in vivo**

PVIgGs were isolated by FPLC protein G affinity chromatography using the FPLC System purchased from Amersham Pharmacia Biotech (Piscataway, NJ) following the manufacturer’s protocol, as detailed elsewhere (17). Minocycline, nicotinamide and cyclosporine A were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). One day old pups delivered by the BALB/c mice purchased from The Jackson Laboratory were used to investigate the effect of mitochondria-protecting drugs on the extent of epidermal acantholysis induced by passive transfer of 1 mg/g/body weight of PVIgG. The pups were injected subcutaneously with PVIgG with or without test drugs and examined 24 hrs later for the extent of epidermal acantholysis, as detailed elsewhere (20). Briefly, the euthanized animals were snap-frozen in liquid nitrogen, cross-sectioned at the umbilicus level, embedded into the OCT compound (Miles Scientific, Naperville, IL),
stained by H&E and evaluated by light microscopy. Five random microscopic fields in each skin section were captured at magnification 10x, using a Macintosh computer attached to the Axiovert 135 inverted microscope. The images were printed, and the extent of acantholysis was computed directly on the prints by measuring the length of the areas in the epidermis in which suprabasal cell detachment spread along more than 4 adjacent basal cells.

Statistical analysis
The data were analyzed using ANOVA against an alpha level of 0.05 and presented as mean ± standard deviation (SD). Graphs were made using GraphPad Prism 5.

RESULTS

Antigen-specificities of MtAbs determined in our proteomic study
Previously, we had demonstrated that PV patients develop autoantibodies that can find their way to mitochondria in KCs and react with a versatile group of mitochondrial proteins, which contributed to PV-like cell detachment in monolayers of human KCs and epidermis of neonatal mice (20). Most recently, we have tested 264 pemphigus and 138 normal control sera on the multiplexed protein array platform containing 701 human genes encompassing many keratinocyte molecules, including 283 mitochondria-associated proteins (8). A detailed analysis of the supplementary data published by us online (8) identified the most common antigens recognized by MtAbs and the percentage of PV patients and healthy people producing such MtAbs (Table 1). Based on the known functions of mitochondrial proteins most commonly targeted in PV, the following mitochondrial pathways might be subject to dysfunction: oxidative phosphorylation, O₂ respiration and production/inactivation of ROS.

PV sera alter mitochondrial O₂ respiration in KCs
To identify the direct effect of MtAbs on O₂ respiration, we performed a Seahorse Bioscience XF24-3 extracellular flux analysis of OCR in KCs treated with PV or normal human sera. While neither of four tested normal serum changed basal mitochondrial respiration, all PV sera significantly increased this mitochondrial function (Fig. 1A, B). The PV sera also significantly increased proton leakage from treated cells (Fig. 1C). These results suggested that MtAbs produced by PV patients increase the ROS production in KCs and reduce the ability of these cells to respond to stress, which can trigger the mitochondria mediated (intrinsic) apoptosis.

PV sera alter ΔΨm in KCs
Exposure of KCs to PV, but not normal, sera caused dramatic changes in the mitochondrial membrane potential, manifested by disappearance of orange-red fluorescence of JC-1 dye with simultaneous increase in green fluorescence of cultured cells (Fig. 2A). A significant drop in the mitochondrial membrane potential in KCs treated with PV sera was confirmed by measurements of ΔΨm using FACS (Fig. 2B). These results indicated that binding of MtAbs to mitochondrial target proteins disrupts the electron transfer chain resulting in loss of electrochemical gradient across the inner membrane.

PV sera increase intracellular ROS production in KCs
When intracellular ROS production was measured by the c-H₂DCFDA-AM dye labeling of cultured KCs, we observed a drastic increase of cell staining in response to treatment by each individual PV serum, compared to normal sera (Fig. 3A). Similar results were obtained in FACS analysis (Fig. 3B), confirming that treatment with PV sera significantly increases the number of c-H₂DCFDA-AM-positive KCs, compared to normal controls. These results indicated that binding of MtAbs to keratinocyte
mitochondria increases intracellular ROS production.

**Mitochondria-protecting drugs abolish deleterious effects of PV sera on mitochondrial O₂ respiration in KCs**

To evaluate direct effects of the mitochondria-protecting drugs minocycline, nicotinamide and cyclosporine A, given alone or in combination, on the KCs exposed to PV serum, we measured OCR in real time in a Seahorse XF Efflux Analyzer, and calculated proton leak. KCs exposed to different PV sera showed differential responses to test drugs (Fig. 4A). Drug combination regimen showed the highest efficacy in abolishing the PV serum-induced elevation of OCR and proton leak (Fig. 4A). These results indicated that while each PV patient's MtAbs display a unique pattern of mitochondrial damage, a combination of mitochondria-protecting drugs exhibits a uniform protective action.

**Treatment of experimental PV in neonatal mice by pharmacologically protecting the mitochondria**

Next, we sought to confirm the therapeutic efficacy of the mitochondria-protecting drugs in the passive transfer model of PV in neonatal BALB/c mice. Co-administration of PVlgG and each of the test drugs abolished the acantholytic activity of PVlgG, albeit with different efficacy (Fig. 4B). A decrease of PVlgG-induced epidermal splitting in mice treated with minocycline or cyclosporine A reached statistical significance (p<0.05). Remarkably, the highest efficacy of mitochondrial protection of the combination of minocycline, nicotinamide and cyclosporine A observed in the mitochondrial assay was consistent with the ability of this combination to almost completely abolish acantholysis in mouse skin (Fig. 4B). Thus, preventing the MtAb-dependent mitochondrial damage protected KCs from PVlgG-induced detachment and skin blistering.

**DISCUSSION**

PV is a complex, multifactorial disease (5, 25). The fact that adsorption of MtAbs from PVlgGs abolished the ability of PVlgGs to induce acantholysis (20), provided strong evidence of the indispensable role of mitochondrial damage in the disease mechanism. In this study, we demonstrated for the first time dramatic changes in the mitochondrial O₂ respiration, dissipation of ΔΨm and drastic increase in the intracellular levels of ROS. These changes can induce intrinsic apoptosis, which is consistent with observations that PVlgG binding to KCs is associated with mitochondrial damage manifested by cytochrome c release and activation of caspase 9 (17-20). Since the mode of mitochondrial damage by MtAbs depends on the biological function of targeted protein, the exact primary mechanism of mitochondrial damage apparently differ from patient to patient, in keeping with striking variations in the disease severity and response to treatment among different PV patients (26). Differential pattern of mitochondrial damage in PV has been also suggested by the fact that the mitochondrial function in KCs exposed to different PV sera showed differential responses to the same mitochondria-protecting drugs (Fig. 4A).

The structural-functional interrelationships of mitochondria-associated proteins targeted by MtAbs (Table 1) indicates that autoantibody binding to even a single mitochondrial target antigen can distort the neatly orchestrated cascade of interactions of enzymes and their substrates, producing a domino effect resulting in the mitochondrial dysfunction. Since MtAbs produced by PV patients can bind their target proteins at both mitochondrial outer and inner membranes, as well as mitochondrial matrix, they can interfere with the biological processes taking place at these locations. Furthermore, since MtAbs recognized both mitochondrial- and nuclear-encoded proteins involved in regulation or execution of specific mitochondrial functions (Table 1), the protein import into the
mitochondria may be also altered, resulting in a pathologic remodeling of mitochondrial proteome.

Results of the present study clearly demonstrated that exposure of KCs to PV serum can disrupt their ΔΨm. This phenomenon can be explained based on the knowledge about involvement of mitochondrial structures in multiple physiological pathways, so that formation of an antigen-antibody complex in one location can influence several biochemical reactions. For example, binding of MtAbs to the complex I proteins, such as NADH dehydrogenase or electron-transfer-flavoprotein targeted in PV (Table 1), can interfere with electron exchange among the carriers and their access to succinate and NADH, and affect their ability to establish a chemiosmotic gradient. Thus, disruption of ΔΨm in KCs exposed to PV sera observed in this study can prevent electron transport (due to MtAb binding one or more electron carriers), which is an early event in apoptosis activated through the mitochondrial pathway (reviewed in (27)).

It is well-established that a delicate balance exists between the moderate ROS production to modulate physiological signaling and the overproduction of ROS that can lead to oxidative stress. ROS detoxification pathways exist to minimize oxidative damage. The presence of anti-superoxide dismutase MtAb in PV patients (Table 1) is in keeping with our observation that preincubation of KCs with PV sera led to excessive ROS production, thus precipitating changes in mitochondrial homeostasis. However, the mitochondria damaged by other types of MtAbs can also lead to ROS increase, creating a pathological process termed the "vicious cycle." For example, impairment in the mitochondrial electron transport that leads to a loss of ΔΨm also can be responsible for an incomplete O2 reduction, resulting in an increment in ROS production that further amplifies the generation of ROS. Such abnormalities can, in turn, trigger the cell death signaling cascade, wherein the executioner caspases cleave the cell adhesion molecules, such as desmosomal cadherins (reviewed in (28)), leading to both cell detachment (acantholysis) and death (apoptosis)—the unique form of keratinocyte demise in PV that we have tentatively termed apoptolysis (21). Further elucidation of the mitochondrial mechanisms of apoptolysis in PV should therefore improve our understanding of disease pathogenesis and facilitate development of personalized therapies based on the pharmacological correction of mitochondrial abnormalities in individual PV patients.

Results of the present study provide first experimental evidence that pharmacological protection of mitochondrial function can prevent skin blistering induced by PVIgG. These results shed light on the mechanism of therapeutic action of minocycline and nicotinamide that have been empirically shown to be effective steroid-sparing agents in PV, as well as of the professional immunosuppressor cyclosporine A. Importantly, reports in the literature testify that in some patients PV can be controlled by the mitochondria-protecting drugs alone without the need for prednisone (29-35). Although these drugs belong to different chemical groups, each exhibiting unique pharmacological effects, they share the mitochondria-protecting activities (Table 2). However, except for empirically chosen combinations of minocycline and nicotinamide, no other combinations of the mitochondria-protecting drugs have been reported in PV. Our results demonstrated synergy among minocycline nicotinamide and cyclosporine A. The combination of drugs that most effectively protected mitochondrial respiration equally effectively antagonized the disease-causing activity of PVIgG in mouse skin. Thus, their synergistic potential is yet to be fully explored.

Since the mitochondria-protecting drugs used in this study are already in use in the treatment of PV patients, the obtained
results translate into clinical practice. There is a strong rationale for the principally new treatment of patients with PV. The existing therapies do not allow one to reliably control acute PV without the systemic corticosteroid prednisone, which, while being life saving, causes serious adverse events, including death, due to the need for its high-dose (eg, 1-2 mg/kg/day) and prolonged (eg, ~18 months) usage to achieve disease control (1,2). Although the incidence of PV is only 1 to 16 per million population per year (36,37), this disease represents a significant burden to health care professionals, and the health care system due to the hazardous side effects of conventional immunosuppressive therapy requiring prolonged and frequent hospitalizations, and high costs of modern treatment regimens (38). By identifying the dose-depended effect, one should be able to increase drug efficacy, because the mitochondria-protecting drugs are being used in PV patients at their suboptimal doses. For example, while nicotinamide is given to PV patients at 1.5-2 g/day, this drug can be safely used at higher doses, like in patients with diabetes or schizophrenia (39). Likewise, cyclosporine A is usually used in PV at 0.5-1.5 mg/kg/day, which is much less compared to the dosages used in transplantology (40). These fact suggest that current suboptimal clinical activity of the mitochondria-protecting drugs used in PV can be improved by identifying their optimal combination through the laboratory screening of patient’s serum prior to treatment. Since the mitochondria-protecting drugs are expected to have different effects in different PV patients, as the mitochondrial pathways targeted by MtAbs are apparently unique to each patient, an individualized regimen will be needed to achieve the optimal therapeutic response.

In conclusion, the integrity of mitochondrial function is fundamental to cell life. Mitochondria are involved in many processes essential for cell survival, such as energy production, redox control, calcium homeostasis and a number of metabolic and biosynthetic pathways. In addition, mitochondria often play an essential role in physiological cell death mechanisms (reviewed in (41)). Results of the present study indicate that binding of PV IgG to mitochondrial targets can disrupt the electron transfer chain resulting in a decline in ATP production, loss of electrochemical gradient across the inner membrane, and reduction in $O_2$ with increased generation of the ROS superoxide, hydrogen peroxide and hydroxyl radical. These novel findings provide a theoretical background for clinical reports of the efficacy of mitochondria-protecting drugs in PV patients. Pharmacological protection of mitochondria and/or compensation of an altered mitochondrial function may therefore become a novel approach to development of safer, ie, non-hormonal, therapies of this severe autoimmune blistering disease.

ACKNOWLEDGEMENT

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FIGURE LEGENDS

Figure 1. Deleterious effects of PV sera on mitochondrial O2 respiration in KCs.
A, Time-course of measurements of OCR in a Seahorse XF bioenergetics assay in Het-1A cells treated with four normal (N1-N4) or PV (P1-P4) sera at the final concentration of 4% vs. no sera (mock), as detailed in Materials and Methods. The data represent mean ± SD of quintuplicate measurements for each individual serum. FCCP is carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone.
B, Mean values of basal respiration calculated from 3 measurements before injection of oligomycin.
C, The proton leak calculated using the OCR measurement after oligomycin injection minus the OCR measurement after rotenone injection.
* = p <0.0001; ** = p <0.0002 compared to mock treatment.

Figure 2. Effects of PV sera on mitochondrial membrane potential (ΔΨm) in KCs.
A, Representative images of the cultured Het-A1 cells treated with PV vs. normal sera, or left untreated (mock), and subjected to evaluation of JC-1 dye staining via fluorescence microscopy, as described in Materials and Methods. The right column displays enlarged images of individual cells from the corresponding low-power images of the left column. Loss of red fluorescence in cells treated with PV serum indicates loss of ΔΨm.
B, FACS measurements of ΔΨm in the Het-1A cells labeled with JC-1 dye. The ratio between green and red fluorescence for mock treated cells was set as 1. * = p <0.0001 compared to mock treatment.

Figure 3. Effects of PV sera on ROS production in KCs.
A, Representative images of cultured Het-A1 cells treated with PV vs. normal sera and subjected to microscopic evaluation of the c-H2DCFDA-AM dye staining via fluorescence microscopy, as described in Materials and Methods. The images were taken with Zeiss AxioPlan II fluorescence microscope using the same exposure time (40 ms). Increased intensity of cells fluorescence due to treatment with PV serum indicates elevated ROS production.
B, FACS analysis of intracellular ROS levels in Het-1A cells labeled with c-H2DCFDA-AM dye. The fluorescence intensity of intact cells (mock) was set as 1. * = p <0.0001 compared to mock.

Figure 4. Mitochondria-protecting drugs abolish effects of MtAbs.
A, Differential effects of mitochondria-protecting drugs against the deleterious effects of PV from different patients. Representative results showing the ability of selective drugs to abolish an increase of OCR and proton leak. Het-1A cells were analyzed in real-time with a Seahorse XF analyzer, as detailed in Materials and Methods. The cells were pretreated with 0.05 mM minocycline (M) ± 10 mM nicotinamide (N) ± 0.1 mM cyclosporine A (C) for 30 min prior to incubation with sera from two PV patients. The untreated KCs (U) served as control. The drugs had no effects on control cells (not shown). * p<0.05 compared to control; # p<0.05 compared to PV sera without drugs.
B, Treatment of experimental PV in neonatal mice by protecting the mitochondria. Neonatal BALB/c mice (n = 3) received a single subcutaneous injection of 1 mg/g of body weight of PVIgG alone or in combination with test drug(s), and the extent of acantholysis in mouse epidermis was measured 24 hrs after injection using a morphometric assays described in Materials and Methods. * p<0.05 compared to PVIgG injected alone.
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Table 1. Mitochondrial Proteins Recognized by MtAbs from PV Patients and Healthy Controls (8).
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Protein name</th>
<th>Localization</th>
<th>Biological process</th>
<th>Function</th>
<th>Frequency in PV vs Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>Pyruvate carboxylase</td>
<td>Matrix</td>
<td>Gluconeogenesis and lipid synthesis</td>
<td>Synthesis of phosphoenolpyruvate from pyruvate.</td>
<td>32% 5%</td>
</tr>
<tr>
<td>PMPCB</td>
<td>Mitochondrial processing peptidase beta subunit</td>
<td>Matrix</td>
<td>Mitochondrial organization</td>
<td>Cleaves presequences from mitochondrial protein precursors.</td>
<td>31% 4%</td>
</tr>
<tr>
<td>PDHA1</td>
<td>Pyruvate dehydrogenase E1 component alpha subunit</td>
<td>Matrix</td>
<td>Glycolysis</td>
<td>Provides the primary link between glycolysis and tricarboxylic acid (TCA) cycle by catalyzing the irreversible conversion of pyruvate into acetyl-CoA.</td>
<td>30% 3%</td>
</tr>
<tr>
<td>FH</td>
<td>Fumarate hydratase (Fumarase)</td>
<td>Mitochondrion</td>
<td>TCA cycle</td>
<td>Catalyses the reversible hydration/dehydration of fumarate to malate.</td>
<td>29% 3%</td>
</tr>
<tr>
<td>MLYCD</td>
<td>Malonyl-CoA decarboxylase</td>
<td>Mitochondrion</td>
<td>Regulation of TCA cycle</td>
<td>Catalyses the conversion of malonyl-CoA into acetyl-CoA and carbon dioxide. Without this enzyme, an excess of malonic acid inhibits TCA cycle.</td>
<td>29% 4%</td>
</tr>
<tr>
<td>CRAT</td>
<td>Carmitine-O-acetyltransferase</td>
<td>Inner membrane</td>
<td>Fatty acid and lipid metabolism</td>
<td>Specific for short chain fatty acids. May be involved in the transport of acetyl-CoA into mitochondria. acetylcholine synthesis.</td>
<td>28% 7%</td>
</tr>
<tr>
<td>MAOB</td>
<td>Amine oxidase (flavin-containing) B</td>
<td>Outer membrane</td>
<td>TCA cycle</td>
<td>Catalyzes the oxidative deamination of biogenic and xenobiotic amines.</td>
<td>27% 5%</td>
</tr>
<tr>
<td>PRODH</td>
<td>Proline oxidase</td>
<td>Matrix</td>
<td>Proline metabolism</td>
<td>Catalyzes the first step in proline degradation.</td>
<td>25% 6%</td>
</tr>
<tr>
<td>FDXR-V2</td>
<td>NADPH:adrenodoxin oxidoreductase</td>
<td>Matrix</td>
<td>Electron transport; cholesteral, lipid and steroid metabolism</td>
<td>The first electron transfer protein in all the mitochondrial P450 systems.</td>
<td></td>
</tr>
<tr>
<td>NDUFA13</td>
<td>NADH dehydrogenase [ubiquinone] 1 alpha subcomplex</td>
<td>Inner membrane</td>
<td>Electron transport; respiratory chain; apoptosis</td>
<td>A subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (complex I). Involved in the interferon/all-trans-retinoic acid induced cell death.</td>
<td>25% 6%</td>
</tr>
<tr>
<td>PDK4</td>
<td>Pyruvate dehydrogenase kinase, isozyme 4</td>
<td>Matrix</td>
<td>Carbohydrate and glucose metabolism</td>
<td>Inhibits the mitochondrial pyruvate dehydrogenase complex, thus contributing to the regulation of glucose metabolism.</td>
<td>24% 6%</td>
</tr>
<tr>
<td>ME3</td>
<td>NADP-dependent malic enzyme, mitochondrial</td>
<td>Matrix</td>
<td>Links the glycolytic and TCA cycles</td>
<td>Generates NADPH for oxidative decarboxylation of malate to pyruvate.</td>
<td>24% 4%</td>
</tr>
<tr>
<td>SOD2</td>
<td>Superoxide dismutase [Mn]</td>
<td>Matrix</td>
<td>Antioxidant defense</td>
<td>Catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide.</td>
<td>23% 8%</td>
</tr>
<tr>
<td>ALDH4A1</td>
<td>Aldehyde dehydrogenase 4 family, member A1</td>
<td>Matrix</td>
<td>Links the urea and TCA cycles</td>
<td>Converts delta-1-pyrroline-5-carboxylate to glutamate.</td>
<td>23% 2%</td>
</tr>
<tr>
<td>NDUFS6</td>
<td>NADH dehydrogenase [ubiquinone] iron-sulfur protein 6; 13 kDa</td>
<td>Inner membrane</td>
<td>Electron transport; respiratory chain</td>
<td>A subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (complex I).</td>
<td>23% 5%</td>
</tr>
<tr>
<td>ETFB</td>
<td>Electron-transfer-flavoprotein, beta protein</td>
<td>Matrix</td>
<td>Electron transport</td>
<td>Transfers electrons to mitochondrial respiratory chain via ETF-ubiquinone oxireductase.</td>
<td>22% 5%</td>
</tr>
<tr>
<td>NDUFA9</td>
<td>NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9; 39 kDa</td>
<td>Matrix</td>
<td>Electron transport; respiratory chain</td>
<td>A subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (complex I).</td>
<td>21% 3%</td>
</tr>
<tr>
<td>TIMM44</td>
<td>Mitochondrial import inner membrane translocase subunit</td>
<td>Inner membrane</td>
<td>Protein transport.</td>
<td>Translocation of transit peptide-containing proteins from the inner membrane into the mitochondrial matrix in an ATP-dependent manner.</td>
<td>20% 3%</td>
</tr>
<tr>
<td>ETFA</td>
<td>Electron-transfer-flavoprotein, alpha protein</td>
<td>Matrix</td>
<td>Electron transport</td>
<td>Transfers electrons to mitochondrial respiratory chain via ETF-ubiquinone oxireductase.</td>
<td>20% 4%</td>
</tr>
<tr>
<td>CYB5B</td>
<td>Cytochrome b5 type B; 21 kDa</td>
<td>Outer membrane</td>
<td>Electron transport</td>
<td>An electron carrier for several membrane bound oxidases.</td>
<td>19% 4%</td>
</tr>
<tr>
<td>ABAT-V1</td>
<td>4-aminobutyrate aminotransferase,</td>
<td>Matrix</td>
<td>Neurotransmitter degradation</td>
<td>Catalyzes the conversion of gamma-aminobutyrate and L-beta-</td>
<td>19% 1%</td>
</tr>
<tr>
<td>Protein</td>
<td>Description</td>
<td>Molecular Weight</td>
<td>Location</td>
<td>Function</td>
<td>% Change</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
<td>------------------</td>
<td>----------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td>NDUFV3</td>
<td>NADH dehydrogenase [ubiquinone] flavoprotein 3; 9 kDa</td>
<td>Mitochondrial; 50 kDa</td>
<td>Inner membrane</td>
<td>Electron transport; respiratory chain</td>
<td>19%</td>
</tr>
<tr>
<td>CPT1B</td>
<td>Cmitine O-palmitoyltransferase 1B</td>
<td></td>
<td>Outer membrane</td>
<td>Fatty acid and lipid metabolism</td>
<td>18%</td>
</tr>
<tr>
<td>ME2</td>
<td>NAD-dependent malic enzyme</td>
<td></td>
<td>Matrix</td>
<td>TCA cycle</td>
<td>18%</td>
</tr>
<tr>
<td>NDUFB10</td>
<td>NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10</td>
<td></td>
<td>Matrix</td>
<td>Electron transport; respiratory chain</td>
<td>17%</td>
</tr>
</tbody>
</table>
Table 2. Drugs Affecting Mitochondrial Stability and Functions Used to Treat PV Patients

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mechanism of protective action on mitochondria</th>
<th>Treatment of PV patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporine A</td>
<td>Inhibits mitochondrial permeability transition pore (mPTP) by preventing the binding of cyclophilin D to pore proteins (42,43).</td>
<td>Used to treat PV patients as an immunosuppressor drug both systemically and topically. Has been effective as a monotherapy in a few PV patients, but most commonly used as a steroid-sparing drug, inducing a prolong remission (29-31,44-47). Partially inhibited signs of experimental PV in mice (48).</td>
</tr>
<tr>
<td>Nicotinamide (NAM), a.k.a. niacinamide, an amide of nicotinic acid. One of two principal forms of the B-complex vitamin, B3.</td>
<td>NAM is a precursor of the coenzyme NAD⁺ used to generate ATP in the mitochondrial electron-transport chain; can increase levels of NAD⁺, NADH, NADP⁺ and NADPH, inhibit poly-ADP-ribose polymerase (PARP), and prevent/reverse the depolarization of the mitochondrial membrane (49). Also, can facilitate the anti-apoptotic nicotinergic signaling (50) by increasing the levels of endogenous cholinomimetic acetylcholine and the α7 nicotinic acetylcholine receptor agonist choline (51-53) and inhibition of the acetylcholine hydrolyzing enzyme acetylcholinesterase (54,55).</td>
<td>Used to treat PV patients both systemically and topically (35), producing steroid-sparing effects. Usually combined with either tetracycline (32-34,56) or minocycline (57).</td>
</tr>
<tr>
<td>Minocycline</td>
<td>Inhibits mPTP by reducing mitochondrial Ca²⁺ uptake, decreases release of mitochondrial pro-apoptotic and increases anti-apoptotic factors, and also modulates the activity of PARP (41).</td>
<td>Used a steroid-sparing drug to treat PV patients alone or in combination with NAM (34,57-60).</td>
</tr>
</tbody>
</table>
**Figure 1.**
Figure 2.
Figure 3.
Figure 4.
Mechanisms of Mitochondrial Damage in Keratinocytes by Pemphigus Vulgaris Antibodies
Mina Kalantari-Dehaghi, Yumay Chen, Wu Deng, Alex Chernyavsky, Steve Marchenko, Ping H. Wang and Sergei A. Grando

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