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Caspase 3 expression and plasma level of Fas ligand as apoptosis biomarkers in inflammatory endotoxemic lung injury

RALUCA-ŞTEFANIA FODOR[1)], ANCA-MEDA GEORGESCU[2)], BIANCA-LIANA GRIGORESCU[3)], ADRIAN-DAN CIOC[4)], MIHALY VERES[5)], OVIDIU-SIMION COTOI[3)], PAL FODOR[6)], SANDA-MARIA COPOTOIU[1)], LEONARD AZAMFIREI[1)]

[1)]*Department of Anesthesiology and Intensive Care, University of Medicine and Pharmacy of Tîrgu Mureş, Romania*

[2)]*Department of Infectious Diseases, University of Medicine and Pharmacy of Tîrgu Mureş, Romania*

[3)]*Department of Physiopathology, University of Medicine and Pharmacy of Tîrgu Mureş, Romania*

[4)]*Clinic of Anesthesiology and Intensive Care, Emergency County Hospital, Tîrgu Mureş, Romania*

[5)]*PhD Student, Department of Anesthesiology and Intensive Care, University of Medicine and Pharmacy of Tîrgu Mureş, Romania*

[6)]*PhD Student, Clinic of Orthopedics and Traumatology, Emergency County Hospital, Tîrgu Mureş, Romania*

Abstract

Objective: The objective of this study is to evaluate if the immunohistochemical expression of a pulmonary apoptosis marker and plasma level of Fas ligand (FasL) correlates with the dose- and time-dependent severity of lung injury, induced by the administration of lipopolysaccharide (LPS) in an endotoxemic rat model. *Materials and Methods*: Our study included 30 male Wistar rats, randomly divided into three groups: one control group (n=6) and two experimental groups (n=12/group), in whom we induced endotoxemia by intraperitoneal injection of progressively increasing doses of LPS (5, 10 mg/kg). We measured FasL plasma levels of the rats at different time points and analyzed the relationships with markers of lung injury. To investigate the level of caspase 3-protein expression, the immunohistochemistry of the lung tissue was assessed. *Results*: The median percentage of caspase 3-stained cells for the 5 mg/kg LPS dose was 0.36%, for the 10 mg/kg LPS dose was 0.4% and for the control group was 0.03% (p<0.0001). The elevated expression levels of caspase 3 were consistent with the altered lung morphologies observed (r_s=0.88). LPS administration in rats resulted in a significant dose-dependent increase in the levels of plasma FasL (p<0.0001). These levels correlated with markers of lung injury: degree of hypoxemia (r_s=-0.42), histological measured lung injury score (r_s=0.72), the density of the caspase 3 staining cells in the immunohistochemistry assessment of apoptosis (r_s=0.81) and with the plasma RAGE (receptor for advanced glycated end-products) values (r_s=0.70). *Conclusions*: Apoptosis is increased in edotoxemia induced lung injury and is likely to contribute to alveolar injury.

Keywords: lipopolysaccharides, lung injury, rat, apoptosis, caspase 3, FasL.

Introduction

Acute respiratory distress syndrome (ARDS), the extreme aspect of lung injury, is a syndrome characterized by a neutrophil-mediated, diffuse alveolo-capillary wall injury along with alveolar and interstitial edema because of increased permeability of pulmonary vasculature. The severity of ARDS depends significantly on the balance between epithelial alveolar and vascular endothelial injuries, and their repair mechanisms [1].

Cells die by one of two processes: apoptosis or non-apoptotic cell death, the traditionally called ‘necrosis’. Apoptosis or programmed cell death is involved in removal of senescent cells and is thought to be essential for the non-injurious resolution of inflammation [2]. The cells involute and die with minimal harm to nearby cells [3]. In contrast, the ‘necrotic’ cell death is characterized by inflammation and widespread damage. Apoptosis in ARDS is fundamental for clearance of neutrophils and removal of overly abundant, type II pneumocytes.

Apoptosis in ARDS

Apoptosis mechanisms are encoded in the chromosomes of all nucleated cells. Two major apoptotic pathways have been defined in mammalian cells: the extrinsic or death receptor pathway and the intrinsic or the mitochondrial pathway. In both pathways, cysteine aspartyl-specific proteases (caspases) are activated and cleave cellular substrates [4].

The extrinsic pathway involves cell surface death receptors belonging to the tumor necrosis factor-receptor (TNF-R) family. TNF receptor-1, CD95/APO-1/Fas, APO-3/DR3/WSL-1/TRAMP/LARD, TRAIL receptor-1/DR4 and TRAIL receptor-2/DR5 [5]. The specific ligands for these receptors belong to the TNF family and include TNF-α, Fas-ligand, lymphotoxin (LT), apo-3 ligand and TNF-related apoptosis inducing ligand (TRAIL) [4]. Receptor activation triggers initiator caspases, like caspases 8 and 10. The extrinsic and intrinsic pathways are intimately connected, and both pathways of apoptosis signaling converge into a common pathway causing the

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activation of effector or executioner caspases 3, 6, and 7 [4]. Executioner caspases orchestrate the appearance of late apoptosis markers such as DNA fragmentation and blobbing of the plasma membrane [6].

The aim of this study is to evaluate the immunohistochemical expression of a pulmonary apoptosis marker and plasma level of Fas ligand in experimental model correlates with the dose- and time-dependent severity of lung injury.

Materials and Methods

Animal care

Our study included 30 male Wistar rats aged 10–12 weeks and weighing 220–260 g, randomly divided into three groups: control group (*n*=6) and two experimental groups (*n*=12/group). All experimental procedures with animals followed the international recommendations for the use and care of animals and all experimental protocols were approved by Institutional Review Board of the University of Medicine and Pharmacy of Tîrgu Mureş, Romania. The animals were acclimatized to the usual laboratory conditions, 14 days before the experiment.

Experimental design

After four hours of stopping nutrition, we administered lipopolysaccharide (lipopolysaccharide from *Escherichia coli* 0111:B4 – Quadratech Diagnostics Ltd.) intraperitoneally, in gradually increasing doses of 5 and 10 mg/kg to experimental subjects included in groups 1 and 2 respectively, for induction of endotoxemia and lung injury. In the control group, the rats were treated the same way as in lipopolysaccharide (LPS) groups, except for the administration of LPS being substituted with an equal volume of normal saline (NS).

Lung injury was quantified in terms of hypoxemic and microscopically changes at 6 hours and 24 hours after LPS administration. Thus, at 6 hours after administration of lipopolysaccharide, we anesthetized the rats in the control group and six animals in the experimental groups with a mixture of Ketamine–Xylazine (2:1; 0.3 mL/rat intraperitoneally). After a thorough dissection, we incanulated abdominal aorta and arterial blood samples were collected in order to determine blood gasometry and to obtain plasma samples by centrifugation (2000 rpm for 10 minutes). The obtained plasma samples were stocked in sterile tubes and kept at -70^{0}C by the time of processing. After blood collection, the rats were sacrificed right away and stamps of lung, tissues were collected. Blood gasometry was measured by using a Gem Premier 4000 Blood Gas Analyzer. At 24 hours after administration of lipopolysaccharide, the rest of the animals (six rats from each experimental group) were subjected to the same procedure.

Apoptosis related marker measured in plasma

We measured Fas-ligand (FasL) plasma levels of the rats included in the control group and the experimental groups, by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Bioassay Technology Laboratory) and evaluated its clinical significance.

Lung histology

Lung tissue fragments were processed by standard histological methods: fixation in buffered formalin (24 hours), embedding in paraffin, sectioning and staining with Hematoxylin-Eosin (HE) and evaluated under light microscopy (Nikon Eclipse E600).

Immunohistochemistry assessment of caspase 3 in rat lungs

To investigate the level caspase 3 protein expressions, the immunohistochemistry of the lung tissue from the rats included in the study was assessed. Briefly, the approximately 3–5 μm thick paraffin-embedded sections were deparaffinized by xylene–ethanol sequence. Antigen retrieval was performed by microwaving the sections at 600 W (four cycles of 5 minutes each) in citrate, at pH 6 and then cooling at room temperature for 30 minutes. Endogenous peroxidase blocking was done by placing the sections with 3% hydrogen peroxide in phosphate-buffered saline (PBS) pH 7.4 for 30 minutes, followed by washing them with PBS pH 7.4 (three times for 5 minutes each). Endogenous Biotin blocking was performed with Avidin–Biotin kit (Life Technologies, code 00-4303) according to the manufacturer's instructions. Each section was non-specifically blocked with 8% bovine serum albumin (BSA) in PBS pH 7.4 and then incubated with the primary antibody: mouse caspase 3 antibody (CPP32 4-1-18) from Pierce Antibodies (code MA1-16843), 1:500 dilution (in PBS pH 7.4), overnight, at 4^{0}C.

Afterward, the sections were washed (three times for 5 minutes each) in PBS pH 7.4 and incubated with the detection system Histofine Simple Stain Rat MAX PO (M) (Nichirei Bioscience, code NIC-414171F) for 30 minutes, at room temperature. The sections were washed again (three times for 5 minutes each) in PBS pH 7.4 and incubated with the chromogen – 3,3'-diaminobenzidine (DAB) solution, instant prepared according to the manufacturer's protocol (Life Technologies, code 00-2114). The reactions were stopped under a light microscope control within maximum 30 seconds from the incubation. The sections were washed with distillated water (three times for 5 minutes each), counterstained with Mayer's Hematoxylin for 3 minutes and washed again for one minute. The sections were than dehydrated in three alcohol baths with increasing concentration of 70°, 90° and 100°, 5 minute per bath and dried at room temperature. The sections were clarified in four successive xylene baths (three times for 5 minutes each, and one hour for the fourth time). Finally, the sections were mounted and dried at room temperature until next day. For negative controls, the primary antibody was replaced by PBS pH 7.4.

Digital morphometry

In order to quantify the degree of activation of cellular caspase 3 in the lungs of the rats included in the study, we used digital morphometry [7]. Digital slides were made with light microscope (Nikon Eclipse E600) using 40× objective.

For each rat, a number of 10 images/specimen was saved from the digital slides in JPG (Joint Photographic Experts Group) format using the 3DHistech Panoramic Viewer. Thus, a total number of 300 digital images were

collected for the study. Images containing bronchioles and bronchi were excluded from the analysis.

Image analysis was performed using ImageJ software, version 1.49v (developed by Wayne Rasband, National Institutes of Health, Bethesda, Maryland, USA) and Java 1.6.0_20 (32 bit) engine, freely available for download. All image processing was performed on an Intel® Core™ I3, 4 GB memory PC running Microsoft 7.

An experienced pathologist used the Cell Counter plugin, to calculate manually the percentage of positive stained cells out of total number of cells (including neutrophils) present on the image. The positive cells were marked with type 1 color and the negative ones with type 2 (Figure 1).

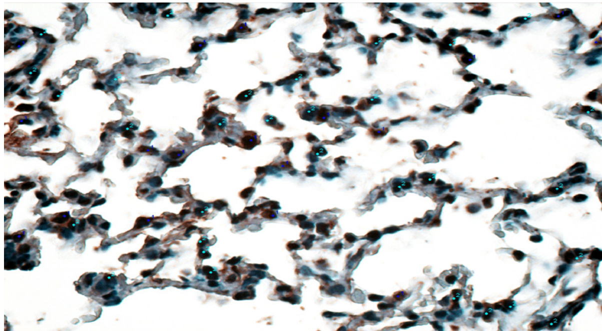

Figure 1 – ***Image analysis method performed using ImageJ software and the Cell Counter plugin, to calculate manually the percentage of positive stained cells out of total number of cells (including neutrophils) present on the image. The positive cells were marked with type 1 color and the negative ones with type 2.***

This method of counting the nuclei is as accurate as the eye can be, so it might be biased by human error. Although it was time consuming, we preferred the manual method to the automated counting method, which offered approximate results. On the images, we found overlapping cells that could have produced false negative results if automated method was to be applied. Therefore, the manual method proved to be more accurate.

Statistical analysis

For statistical analysis, we used SPSS *ver.* 20 (Chicago, IL, USA). For non-normally distributed data, we used non-parametric tests, and for normally distributed data, we used parametric tests. Normally distributed data was reported as means and standard deviations while non-normally distributed data was reported as medians and quartiles. The Mann–Whitney *U*-test was used to compare groups. Spearman's correlation coefficient was used to analyze correlations between parameters. A value of $p<0.05$ was considered statistically significant.

Results

Arterial blood gas analysis, evaluation of epithelial injury and microscopically observations of lung tissue

We have characterized these parameters in a previous study [8].

Briefly, we found that arterial partial pressure of oxygen (PaO_2) in the LPS groups, at six hours, was significantly reduced compared to that in the control group. In the early stage, the severity of hypoxemia correlated with the gradually increasing doses of LPS administered, with a PaO_2 lower mean value of 70.67±6.31 mmHg. At 24 hours, hypoxemia improved most in the group of rats, which received smaller LPS doses.

A variety of specific biomarkers for epithelial injury during ARDS have been proposed, with receptor for advanced glycation end-products (RAGE) the most recently promoted. We found that elevated RAGE plasma levels correlated with increasing dose of LPS administered.

We examined the histology of lung tissues of all the rats by HE staining. The injury progressed with increased time of exposure to LPS. None of the LPS treated groups developed severe fibrin exudation into alveolar space, with the presence of typical hyaline membranes. A pathologist blinded to the treatment groups, quantified for each rat a lung injury score according to the guidelines provided by *American Thoracic Society*. The injury became more severe with increased dose and time of exposure to LPS.

Apoptosis-related marker measured in serum

A Shapiro–Wilk's test and a visual inspection of the histograms, Q–Q plots, were employed to determine the FasL normality distribution within different dosage groups. For all groups the Shapiro–Wilk's test showed the data was not normally distributed and skewed (5 mg/kg group, $p=0.006$; 10 mg/kg group, $p<0.0001$; control group, $p=0.015$). The homogeneity of variance was tested with the non-parametric Levene's test ($p=0.935$). Because that the data was not normally distributed within groups, we compared the groups using the Kruskal–Wallis test. The test showed a significant difference between different dosage groups ($p<0.0001$) in terms of FasL mean rank values. The reported chi-square (χ^2) value for the test was 29.6 with an effect size estimate eta-square (η^2) of 84.7%, which means that 84.7% in FasL variability is accounted for dosage group (higher the dosage, higher the FasL values). For *post-hoc* analysis, we wanted to see if there is a significant difference between specific dosage groups (5 mg/kg *vs.* 10 mg/kg). Between those two dosage groups, the mean FasL rank difference was 6.75 *vs.* 18.25 with a significance value $p<0.0001$ and a η^2 of 69% (Figure 2). Therefore, we conclude that increasing the LPS dosage will produce a statistically significant higher FasL plasma concentration.

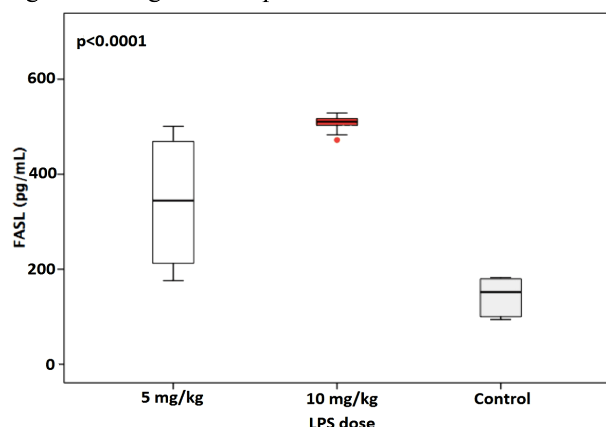

Figure 2 – ***Median FasL values at different LPS dosage. The median FasL value for the 5 mg/kg group was 344.5 pg/mL, for the 10 mg/kg 510.5 pg/mL, and for the control group 152 pg/mL, with a p<0.0001.***

The change in FasL in both groups (5 mg/kg *vs.* 10 mg/kg) related to time was analyzed using the Mann–Whitney *U*-test (Figure 3). In our study, the plasmatic levels of FasL increased significantly with the increasing dose of LPS administered in a time dependent fashion.

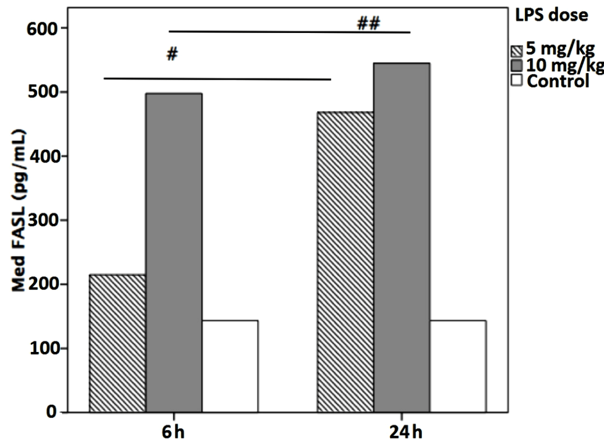

Figure 3 – ***The median difference in FasL values at different LPS dosage related to time. There was a statistical significant change of FasL in both LPS groups from 6 hours to 24 hours (#p=0.004, ##p=0.03). The median values for the 5 mg/kg LPS dose at 6 h was 215 pg/mL vs. 468 pg/mL at 24 h, with a control value of 143 pg/mL. The median values for the 10 mg/kg LPS dose at 6 h was 498 pg/mL vs. 545 pg/mL at 24 h.***

Immunohistochemistry assessment of caspase 3 in rat lungs

Immunohistochemical staining of serial lung tissue sections included in the study demonstrated that the expression of caspase 3 was up-regulated mainly in alveolar wall cells with increased dose and time of exposure to LPS (Figure 4).

Digital morphometry

Figures 5 and 6 show image analysis data for the control group and the experimental groups. Expression of caspase 3 in the lung tissue was quantified as the percentage of positive stained cells out of total number of cells (including neutrophils) present on the image. The experimental groups showed significantly higher expression of caspase 3 in the lung tissue with increased dose and time of exposure to LPS.

Clinical correlations

The FasL plasma level was negatively correlated with the PaO_2 levels (r_s=-0.42, p=0.041), meaning that higher degree of hypoxemia (indicated by lower levels of PaO_2) are associated with higher FasL plasma levels.

The FasL plasma level was positively correlated with the plasma RAGE values (r_s=0.70, p=0.011), meaning higher epithelial dysfunction is associated with higher FasL plasma values.

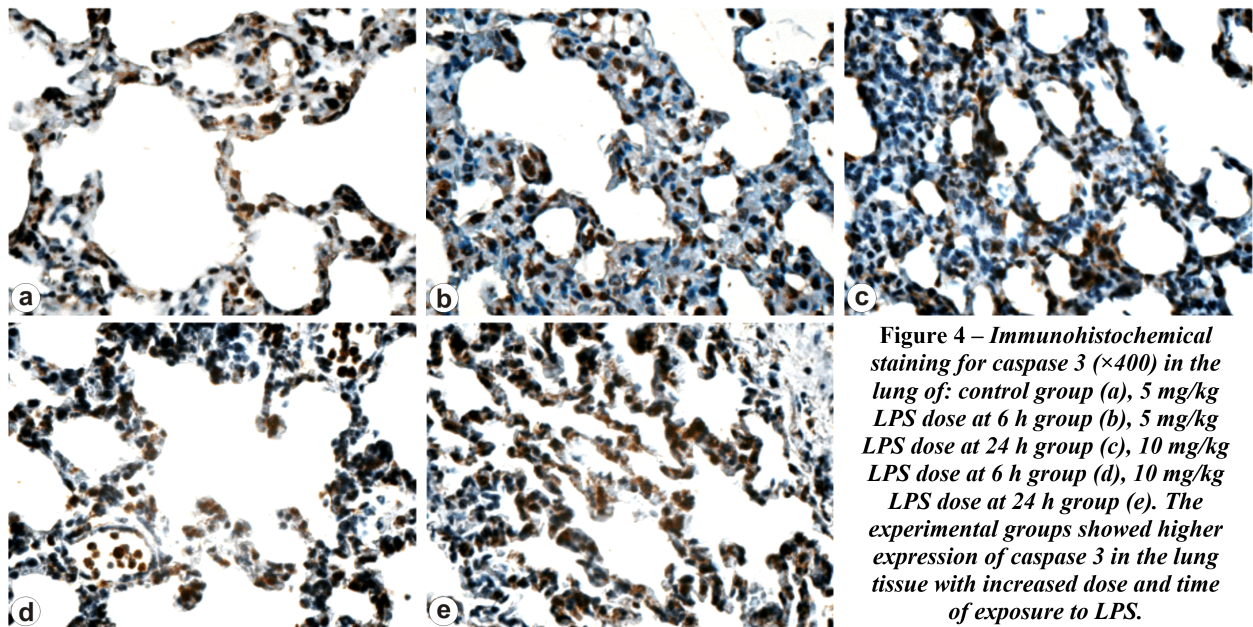

Figure 4 – ***Immunohistochemical staining for caspase 3 (×400) in the lung of: control group (a), 5 mg/kg LPS dose at 6 h group (b), 5 mg/kg LPS dose at 24 h group (c), 10 mg/kg LPS dose at 6 h group (d), 10 mg/kg LPS dose at 24 h group (e). The experimental groups showed higher expression of caspase 3 in the lung tissue with increased dose and time of exposure to LPS.***

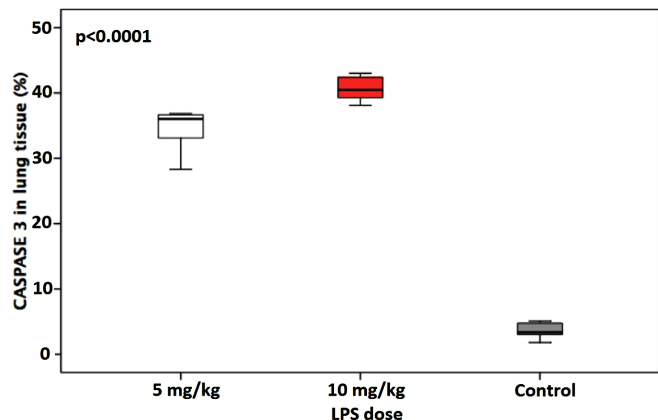

Figure 5 – ***Digital morphometry: quantitative assessment of immunohistochemical staining for caspase 3 at different LPS dosage. The median percentage of caspase 3-stained cells for the 5 mg/kg LPS dose was 0.36%, for the 10 mg/kg LPS dose was 0.4% and for the control group was 0.03%, with a p<0.0001.***

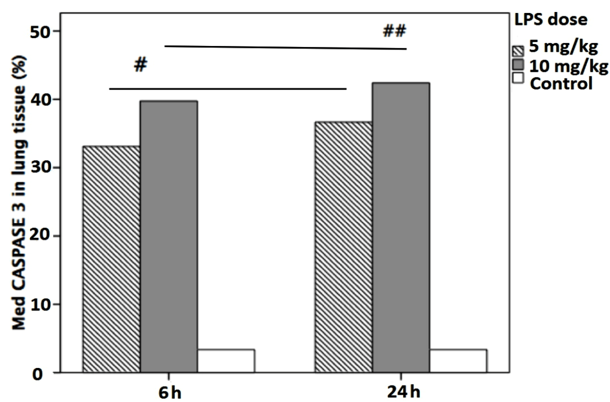

Figure 6 – ***Digital morphometry: quantitative assessment of immunohistochemical staining for caspase 3 at different LPS dosage related to time. There was a statistical significant change of median percentage of caspase 3-stained cells in both groups from 6 h to 24 h (#p=0.004, ##p=0.04).***

The FasL plasma level was positively correlated with our histological measured lung injury score (r_s=0.72, p<0.0001), meaning that higher lung injury scores were associated with higher FasL plasma values.

The density of the caspase 3 staining cells in the immunohistochemistry assessment of apoptosis was positively correlated with the FasL plasma levels (r_s=0.81, p<0.0001). Caspase 3 was also negatively correlated with the PaO_2 level (r_s=-0.83, p<0.0001) and positively correlated with the lung injury score (r_s=0.88, p<0.0001). Caspase 3 staining was quantified as the percentage of positive stained cells out of total number of cells (including neutrophils) present on the image.

Discussion

Recent findings indicate that apoptosis contributes to ARDS pathogenesis [4] in two ways: by delaying neutrophil apoptosis and by enhancing endothelial/epithelial cell apoptosis [9].

Neutrophils apoptosis

Removal of activated neutrophils by apoptosis is a significant mechanism by which the inflammatory response is appropriately terminated [10]. Rapid apoptosis is a characteristic of neutrophils, but in ARDS neutrophils apoptosis is markedly decreased. Neutrophils, once mature, exhibit a constitutive form of programmed cell death with a life span between 6 to 12 hours in circulation. Under normal circumstances, activated neutrophils are eliminated fairly quickly once the invading pathogen has been cleared [11]. Several inflammatory agents are reported to be involved in inhibiting neutrophil apoptosis, including LPS, TNF-α, interleukin (IL)-2, IL-8, granulocyte colony-stimulating factor (G-CSF), granulocyte/macrophage colony-stimulating factor (GM-CSF), and growth-related oncogene-α (GRO-α) [12]. The delayed apoptotic response provides neutrophils with a longer life span, which, in turn, allows them to accumulate at local tissue sites of inflammation/infection [13, 14]. Neutrophil apoptosis returns to normal in the resolution of ARDS.

Alveolar cell apoptosis

Under physiological conditions, the epithelial barrier is less permeable compared to the endothelial barrier. The destruction of its integrity prompts a progressive influx of protein rich fluid into the alveoli [11]. Apoptosis of lung epithelial cells represents a potentially important mechanism contributing to the loss of this cell type in the development of lung injury [4]. There is evidence that both basic apoptotic pathways are activated in ARDS [4, 15–17], but the relative contribution of apoptosis or necrosis in epithelial cells death is unknown [17].

In sepsis, besides direct epithelial necrosis caused by mechanical factors, local ischemia, or bacterial products in the airspaces [4], the Fas/Fas ligand (FasL) system seems to play a central role in apoptosis of the lung epithelial cells [18]. This system is comprised of the cell membrane surface receptor Fas and its natural ligand (FasL). FasL bearing the receptor Fas, initiates the death receptor pathway by activating a series of caspase cascades resulting in apoptosis [19]. Activation of caspase 3 is a central event in the execution of caspase-dependent apoptosis [20].

Fas and FasL exists as membrane bound and soluble forms. Fas antigen, also called APO-1 or CD95, is expressed on many cells, including lymphocytes, neutrophils, and monocytes [4]. In the lung, Fas has been found on the surface of alveolar and bronchial epithelial cells, Clara cells, alveolar macrophages, and myofibroblasts. FasL is expressed on cytotoxic T-lymphocytes (CTLs), activated neutrophils, monocytes, eosinophils, platelets, and alveolar and airway epithelial cells [4].

Soluble Fas (sFas) is produced by alternative splicing of mRNA [4, 21], whereas soluble FasL (sFasL) is generated by cleavage of membrane-bound FasL by metalloproteinase [4, 22]. sFas inhibits cell apoptosis by competing with membrane-bound Fas receptors to bind to FasL [12].

Another potential pathway of lung injury by neutrophils is represented by the release of sFasL that causes apoptosis of alveolocytes type two [4].

The levels of the pro-apoptotic and anti-apoptotic mediators could aid prognosis and staging of the injuries in ARDS. Regulating the activity of the apoptotic cell signaling proteins might be a key element in the pathophysiology of the ARDS, with clinical consequences in the therapeutic particularization (anti- or pro-apoptotic) and the chance of improving the outcome. For this reason, it is necessary to correlate the tissue and plasma expression of the apoptotic markers with clinical and histopathological changes of the lung injury.

Lipopolysaccharide (LPS) has previously been used to induce apoptotic changes including internucleosomal DNA fragmentation and activation of a caspase. It is believed that the method of inducing apoptosis with lipopolysaccharides mimics partially the cellular response to a pathogenic host [23, 24].

The objective of this study is to evaluate if the immunohistochemical expression of a pulmonary apoptosis markers correlates with the dose- and time-dependent severity of lung injury, induced by the administration of LPS in an edotoxemic rat model.

An animal model of acute lung injury stated that following intravenous administration of LPS, the capillary endothelium is the initial site of injury [25]. The cellular

injury induced by LPS appears to be related to apoptosis, as systemic treatment with the broad-spectrum caspase inhibitor Z-VAD-fmk blocks apoptosis and improves survival in mice treated with intravenous LPS [26]. Apoptosis of endothelial cells develops rapidly following administration of LPS and precedes other tissue damage [25], culminating with lung epithelial cells apoptosis in case of ARDS.

Experimental studies show that low levels of lung injury are associated with high levels of apoptosis, whereas increased lung injury is associated with decreased apoptosis and increased necrosis [18].

We found an increased level of active caspase 3, in the rat lungs after exposure to LPS, which became more prominent with time. The elevated expression levels of caspase 3 were consistent with the altered lung morphologies observed. These results indicate apoptosis might be involved in epithelial damage during early stages of lung injury. Our study is in agreement with previous studies that have demonstrated that apoptosis is increased in endotoxemic induced lung injury and is likely to contribute to alveolar injury [27, 28].

Pires-Neto *et al.* (2013) studied the index of airway epithelial apoptosis assessed immunohistochemichally with caspase 3 expression, Fas/FasL epithelial expression, and the TUNEL assay. However, their research showed no evidence that apoptosis is a major mechanism of airway epithelial cell death in patients with ARDS [18].

Our endotoxemia rat model mimics a very acute phase of lung injury as blood and histology samples were extracted after 6 hours and 24 hours after LPS intraperitoneally injection. Therefore, the severity of damage might have been lower than in humans with ARDS. In the previously mentioned study, the epithelial necrosis seems to be an important mechanism of airway epithelial cell death due to the severity of acute lung injury [18].

The rationale that the Fas/FasL system has a potential role in airway epithelial apoptosis is based on the observation that bronchoalveolar lavage fluids obtained from patients with ARDS induced distal lung epithelial cell apoptosis and that this apoptosis was inhibited by blocking the Fas/FasL system [18].

Soluble FasL (sFasL) is found in the bronchoalveolar lavage fluids obtained from patients with ARDS, where levels correlate with the severity of disease [29]. Increased immunoexpression of Fas and FasL in alveolar epithelial cells in patients who died of ARDS has also been demonstrated [30]. A subsequent study that measured soluble Fas (sFas) and sFasL the edema fluids of ARDS showed that sFas, but not sFasL, levels were related to the severity of organ dysfunction [30]. The ratio between pro- and anti-apoptotic proteins might determine the susceptibility of cells to an apoptotic death signal [20].

The concentrations of both sFas and sFasL were higher in the pulmonary edema fluid of patients with ARDS compared to simultaneous plasma samples, indicating local release in the lung [30]. Soluble FasL accumulates at sites of tissue inflammation and has the potential to initiate apoptosis of epithelial lung cells, leukocytes, and other parenchymal cells [4].

Most of the studies published in the literature with regard to apoptosis in lung injury, evaluated the apoptosis related mediators from the site of injury, *i.e.*, bronchoalveolar lavage fluid and pulmonary tissue. In clinical practice, these biological probes are not always readily available. We tried to verify if FasL plasma levels correlate with pulmonary apoptosis and the severity of lung injury. In our study, LPS administration in rats resulted in a significant dose-dependent increase in the FasL plasma levels.

In analyzing the relationships with markers of lung injury, the FasL plasma levels were correlated with all the studied parameters, meaning the oxygenation status (PaO_2), the density of the caspase 3 staining cells in the immunohistochemistry assessment of apoptosis, the histologically measured lung injury score and epithelial dysfunction measured as plasma RAGE level. This implies that FasL might play a central role in the extent of apoptotic epithelial injury.

In terms of concentrations, sFasL may not be sufficient as a single major contributor in the development of lung injury. Its soluble forms only constitute a small portion of the Fas/FasL system, so they could not account for all of the apoptotic events in the milieu of injured alveoli [10]. Recent studies suggest that activation of Fas serves not only to induce apoptosis, but also to induce the secretion of cytokines and chemokines by a variety of cell types [11].

We acknowledge several limitations of our study. Induction of endotoxemia using LPS is a known experimental model, but LPS treatment in rats does not cause the severe endothelial and epithelial injury that occurs in humans with ARDS. Also, our lung injury model mimics a very acute phase, because blood and histology samples were extracted after 6 hours and 24 hours after LPS intraperitoneal injection. The small size groups included in the study limit the power of the study. We also did not measure the bronchoalveolar lavage fluid levels of the studied markers. The data would have been more meaningful if the bronchoalveolar lavage fluid levels had been measured and compared with the plasma levels.

Further studies should be conducted for better understanding of the pathogenic basis of apoptosis in ARDS and factors governing cell susceptibility. Modulation of apoptosis in a cell-, time-, and location-specific manner might be an effective therapeutic strategy against inflammatory lung diseases.

Conclusions

The present study demonstrated that apoptosis is increased in endotoxemic induced lung injury and is likely to contribute to alveolar injury. The elevated expression levels of caspase 3 in the rat lungs after exposure to LPS were consistent with the altered lung morphologies observed. In the present study, LPS administration in rats resulted in a significant dose-dependent increase in the levels of plasma FasL. These levels correlated with all the studied markers of lung injury so FasL might play a central role in the extent of apoptotic epithelial injury.

Conflict of interests

The authors report no conflict of interests.

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References

[1] Zhai R, Gong MN, Zhou W, Thompson TB, Kraft P, Su L, Christiani DC. Genotypes and haplotypes of the VEGF gene are associated with higher mortality and lower VEGF plasma levels in patients with ARDS. Thorax, 2007, 62(8):718–722.

[2] Fialkow L, Fochesatto Filho L, Bozzetti MC, Milani AR, Rodrigues Filho EM, Ladniuk RM, Pierozan P, de Moura RM, Prolla JC, Vachon E, Downey GP. Neutrophil apoptosis: a marker of disease severity in sepsis and sepsis-induced acute respiratory distress syndrome. Crit Care, 2006, 10(6):R155.

[3] Kam PCA, Ferch NI. Apoptosis: mechanisms and clinical implications. Anaesthesia, 2000, 55(11):1081–1093.

[4] Galani V, Tatsaki E, Bai M, Kitsoulis P, Lekka M, Nakos G, Kanavaros P. The role of apoptosis in the pathophysiology of Acute Respiratory Distress Syndrome (ARDS): an up-to-date cell-specific review. Pathol Res Pract, 2010, 206(3):145–150.

[5] Gravestein LA, Borst J. Tumor necrosis factor receptor family members in the immune system. Semin Immunol, 1998, 10(6): 423–434.

[6] Dejean LM, Ryu SY, Martinez-Caballero S, Teijido O, Peixoto PM, Kinnally KW. MAC and Bcl-2 family proteins conspire in a deadly plot. Biochim Biophys Acta, 2010, 1797(6–7):1231–1238.

[7] Mezei T, Szakács M, Dénes L, Jung J, Egyed-Zsigmond I. Semiautomated image analysis of high contrast tissue areas using hue/saturation/brightness based color filtering. Acta Medica Marisiensis, 2010, 57(6):679–684.

[8] Fodor RŞ, Georgescu AM, Cioc AD, Grigorescu BL, Cotoi OS, Fodor P, Copotoiu SM, Azamfirei L. Time- and dose-dependent severity of lung injury in a rat model of sepsis. Rom J Morphol Embryol, 2015, 56(4):1329–1337.

[9] de Souza PM, Lindsay MA. Apoptosis as a therapeutic target for the treatment of lung disease. Curr Opin Pharmacol, 2005, 5(3):232–237.

[10] Bordon J, Aliberti S, Fernandez-Botran R, Uriarte SM, Rane MJ, Duvvuri P, Peyrani P, Morlacchi LC, Blasi F, Ramirez JA. Understanding the roles of cytokines and neutrophil activity and neutrophil apoptosis in the protective *versus* deleterious inflammatory response in pneumonia. Int J Infect Dis, 2013, 17(2):e76–e83.

[11] Perl M, Lomas-Neira J, Chung CS, Ayala A. Epithelial cell apoptosis and neutrophil recruitment in acute lung injury – a unifying hypothesis? What we have learned from small interfering RNAs. Mol Med, 2008, 14(7–8):465–475.

[12] Lee KS, Choi YH, Kim YS, Baik SH, Oh YJ, Sheen SS, Park JH, Hwang SC, Park KJ. Evaluation of bronchoalveolar lavage fluid from ARDS patients with regard to apoptosis. Respir Med, 2008, 102(3):464–469.

[13] Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS Jr. NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science, 1998, 281(5383):1680–1683.

[14] Hadade A, Ionescu D, Mocan T, Necula A, Cristea V. Total intravenous *versus* inhalation anesthesia in patients undergoing laparoscopic cholecystectomies. Effects on two proinflammatory cytokines serum levels: IL-32 and TNF-alfa. J Crit Care Med, 2016, 2(1):44–50.

[15] Hashimoto S, Kobayashi A, Kooguchi K, Kitamura Y, Onodera H, Nakajima H. Upregulation of two death pathways of perforin/granzyme and FasL/Fas in septic acute respiratory distress syndrome. Am J Respir Crit Care Med, 2000, 161(1): 237–243.

[16] Kitamura Y, Hashimoto S, Mizuta N, Kobayashi A, Kooguchi K, Fujiwara I, Nakajima H. Fas/FasL-dependent apoptosis of alveolar cells after lipopolysaccharide-induced lung injury in mice. Am J Respir Crit Care Med, 2001, 163(3 Pt 1):762–768.

[17] Serrao KL, Fortenberry JD, Owens ML, Harris FL, Brown LA. Neutrophils induce apoptosis of lung epithelial cells *via* release of soluble Fas ligand. Am J Physiol Lung Cell Mol Physiol, 2001, 280(2):L298–L305.

[18] Pires-Neto RC, Morales MM, Lancas T, Inforsato N, Duarte MI, Amato MB, de Carvalho CR, da Silva LF, Mauad T, Dolhnikoff M. Expression of acute-phase cytokines, surfactant proteins, and epithelial apoptosis in small airways of human acute respiratory distress syndrome. J Crit Care, 2013, 28(1):111.e9–111.e15.

[19] Han F, Luo Y, Li Y, Liu Z, Xu D, Jin F, Li Z. Seawater induces apoptosis in alveolar epithelial cells *via* the Fas/FasL-mediated pathway. Respir Physiol Neurobiol, 2012, 182(2–3):71–80.

[20] Mou H, Zheng Y, Zhao P, Bao H, Fang W, Xu N. Celastrol induces apoptosis in non-small-cell lung cancer A549 cells through activation of mitochondria- and Fas/FasL-mediated pathways. Toxicol *In Vitro*, 2011, 25(5):1027–1032.

[21] Cheng J, Zhou T, Liu C, Shapiro JP, Brauer MJ, Kiefer MC, Barr PJ, Mountz JD. Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. Science, 1994, 263(5154):1759–1762.

[22] Tanaka M, Itai T, Adachi M, Nagata S. Downregulation of Fas ligand by shedding. Nat Med, 1998, 4(1):31–36.

[23] Vereker E, Campbell V, Roche E, McEntee E, Lynch MA. Lipopolysaccharide inhibits long term potentiation in the rat dentate gyrus by activating caspase-1. J Biol Chem, 2000, 275(34):26252–26258.

[24] Tsuji T, Asano Y, Handa T, Honma Y, Ichinose Y, Yokochi T. Induction of apoptosis in lymphoid tissues of mice after intramuscular injection of enterotoxigenic *Escherichia coli* enterotoxin. Immunobiology, 2010, 201(3–4):377–390.

[25] Matute-Bello G, Frevert CW, Martin TR. Animal models of acute lung injury. Am J Physiol Lung Cell Mol Physiol, 2008, 295(3):L379–L399.

[26] Kawasaki M, Kuwano K, Hagimoto N, Matsuba T, Kunitake R, Tanaka T, Maeyama T, Hara N. Protection from lethal apoptosis in lipopolysaccharide-induced acute lung injury in mice by a caspase inhibitor. Am J Pathol, 2000, 157(2):597–603.

[27] Z'graggen BR, Tornic J, Müller-Edenborn B, Reyes L, Booy C, Beck-Schimmer B. Acute lung injury: apoptosis in effector and target cells of the upper and lower airway compartment. Clin Exp Immunol, 2010, 161(2):324–331.

[28] Chian CF, Chiang CH, Chuang CH, Liu SL. Inhibitor of nuclear factor-κB, SN50, attenuates lipopolysaccharide-induced lung injury in an isolated and perfused rat lung model. Transl Res, 2014, 163(3):211–220.

[29] Matute-Bello G, Liles WC, Steinberg KP, Kiener PA, Mongovin S, Chi EY, Jonas M, Martin TR. Soluble Fas ligand induces epithelial cell apoptosis in humans with acute lung injury (ARDS). J Immunol, 1999, 163(4):2217–2225.

[30] Albertine KH, Soulier MF, Wang Z, Ishizaka A, Hashimoto S, Zimmerman GA, Matthay MA, Ware LB. Fas and fas ligand are up-regulated in pulmonary edema fluid and lung tissue of patients with acute lung injury and the acute respiratory distress syndrome. Am J Pathol, 2002, 161(5):1783–1796.

Corresponding author

Anca-Meda Georgescu, Associate Professor, MD, PhD, Department of Infectious Diseases, University of Medicine and Pharmacy of Tîrgu Mureş, 48 Gheorghe Marinescu Street, 540136 Tîrgu Mureş, Romania; Phone +0741–052 308, e-mail: ancameda.georgescu@umftgm.ro

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