Acute lung injury in children: from viral infection and mechanical ventilation to inflammation and apoptosis

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Active Caspase-3 in Lung Epithelium of Children with ARDS

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ABSTRACT

The objective of this observational study was to investigate the extent of active caspase-3 immunostaining in lung epithelial cells in children with acute respiratory distress syndrome (ARDS). We performed double immunohistochemistry for cleaved caspase-3 and (pan) cytokeratin in lung tissues obtained at autopsy of sixteen children who died with ARDS and diffuse alveolar damage. Spectral imaging was used for the quantification of immunohistochemistry co-localization of these markers. We found a wide range in the percentage of alveolar epithelial cell surface area with positive active caspase-3 staining in the lungs of children with ARDS (from 1 up to almost 20%). The degree of caspase-3 immunostaining in epithelial cells positively correlated with age. We conclude that there is a high variability in the extent of classical apoptosis in lung epithelial cells in pediatric ARDS, potentially in part dependent on age.
INTRODUCTION

Acute respiratory distress syndrome (ARDS) is a major cause of death among intensive care patients worldwide. ARDS is associated with marked (neutrophilic) alveolitis and enhanced lung permeability due to endothelial and epithelial cell dysfunction. An important mechanism of epithelial injury in ARDS is the activation of specific pro-apoptotic pathways in lung epithelial cells \(^1\). Patients who died with ARDS have increased markers of apoptosis, such as cleaved caspase-3, in cells in the alveolar walls \(^2\). Furthermore, several animal studies modeling both indirect and direct causes of ARDS have shown that enhanced lung cell apoptosis is associated with leakage of serum proteins into the lungs and histopathological alterations, which may be attenuated by inhibiting pro-apoptotic pathways \(^3-5\).

So far, studies investigating classical apoptosis by active caspase-3 in lung tissues of ARDS patients have not included specific immunostainings of lung cells, and thus remain limited in the interpretation of cellular distribution of this marker. The purpose of the present brief report was to study the extent of active caspase-3 immunolocalization specifically in lung epithelial cells in pediatric ARDS. To this aim, we performed double immunohistochemistry for active caspase-3 and (pan)cytokeratin in lung tissue sections of sixteen children who died with ARDS and diffuse alveolar damage. Visual detection of co-localizing double staining by light microscopy by eye can be compromised by the loss of color contrast, especially in diffuse injured tissues. Therefore, to quantify co-localization we used the technique of spectral imaging, which can unmix multicolor microscopy into individual images based on the detection of their spectral characteristics \(^6\).

METHODS

Patients

Formalin-fixed, paraffin-embedded lung tissue samples from autopsy of 16 mechanically ventilated patients with ARDS who died after admission to the Pediatric Intensive Care Unit (PICU) in the period 1990-2009 were obtained from the Department of Pathology of the Academic Medical Center Amsterdam and the University Medical Center Utrecht, The Netherlands. All selected patients met the American European Consensus Conference criteria of ARDS \(^7\). The lung tissue cubes were obtained by standard hospital autopsy procedures and fixed in buffered formalin for 24 hr. The need for written informed consent was waived in consultation with the medical ethical research committees in both medical centers.

Immunohistochemistry

Of each lung tissue sample 5-μm-thick sections were cut for double-staining for active caspase-3 and cytokeratin. Sections were xylene-deparaffinized, rehydrated in an alcohol
series, and treated for 20 minutes with 0.3% H$_2$O$_2$ in methanol to block endogenous peroxidase activity. Heat induced antigen retrieval was performed in Tris-EDTA (pH 9.0) at 98°C. After washing in tap water, the sections were treated with Ultra V Block (Immunologic, Duiven, The Netherlands) to block non-specific binding sites. Primary antibody labeling was performed with a cocktail of rabbit anti-human cleaved caspase-3 (Asp175) mAb (Cell Signaling, Danvers, MA) and mouse anti-human cytokeratin 1-8, 10, 13-16 and 19 mAb (AE1/AE3 clone, Dako, Glostrup, Denmark), followed by secondary labeling with a cocktail of anti-rabbit IgG HRP polymer and anti-mouse AP polymer (Immunologic). Visualization of AP activity was performed with AP Substrate Kit (Vector®Blue, Vector Laboratories, Burlingame, CA) and HRP activity was detected with Peroxidase Substrate Kit (Vector®NovaRED, Vector Laboratories). The sections of all patients were stained in the same experiment.

**Quantification**

Both the cleaved caspase-3 and cytokeratin staining have cytoplasmic localization. Detection of co-localization of the Vector®NovaRED and Vector®Blue signals was performed by spectral imaging, as described previously in detail. Per section 5 random digital images containing alveolar structures at moderate (200x) magnification were taken by a Leica® microscope using Nuance 2.8.0. software (CRi; Woburn, MA). After loading the spectral characteristics containing the individual spectra of Vector®NovaRED and Vector®Blue, the Nuance software was used to unmix the spectra in these images. The spectrum of erythrocytes and non-specific staining was filtered out to avoid overlap. For quantification the co-localization tool of the Nuance 2.8.0. software was used to calculate the percentage of pixels with positive cytokeratin signal co-localizing with positive cleaved caspase-3 signal per image.

The same threshold and spectral settings were used for analysis in all images. Observer agreement of signal co-localizations was checked in all images. In ten random 200x images we visually counted the number of double positive cells to assess the degree of correlation with the spectral imaging quantification method.

**Statistical analysis**

Not normally distributed data are shown as box plots including median, interquartile range and full range, and were analyzed by using the Mann-Whitney U test for differences between groups. Spearman’s $r$ was calculated to assess correlation.

**RESULTS**

Patient characteristics are presented in Table 1. Cause of death was multiple organ dysfunction (n=9, including central nervous system failure: n=4), respiratory failure (n=6) or circulatory failure (n=1). All patients had been treated with supplemental oxygen and mechanical ventilation. Two patients had been treated with high frequency oscillatory ventilation.
at some point during admission. The lung histopathology of the patients showed diffuse alveolar damage, with alterations such as (neutrophilic) alveolitis and evidence of endothelial/epithelial dysfunction (e.g. capillary congestion, intra-alveolar protein-rich edema and protein

Table 1. Patient characteristics (n=16).

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<table>
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<tr>
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<tr>
<td>Age; year (SE)</td>
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<tr>
<td>Male; number of patients (%)</td>
<td>9 (56)</td>
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<tr>
<td>Duration of mechanical ventilation; days (SE)</td>
<td>9 (2.3)</td>
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<td>Clinical disorders; number of patients (%)</td>
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<tr>
<td>aspiration</td>
<td>4 (25)</td>
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<tr>
<td>other/unknown</td>
<td>4 (25)</td>
</tr>
<tr>
<td>Indirect: sepsis</td>
<td>4 (25)</td>
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<tr>
<td>Underlying condition; number of patients (%)</td>
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<tr>
<td>(Auto)-immunology</td>
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</tr>
<tr>
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<tr>
<td>leucopenia¹</td>
<td>2 (13)</td>
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¹ WBC < 500 x 10⁶ / L. SE: standard error of mean

Figure 1. A-C, lung tissue sections of ARDS patients stained with haematoxylin and eosin (magnification 100x) showing changes characteristic of exudative ARDS (*protein-rich alveolar exudates) (A), hyaline membranes (arrow) (B), and marked fibroproliferative changes (C). D-F, Immunohistochemistry for cleaved (active) caspase-3 (magnification 500x) showing the wide range of positive staining in lung tissues sections of patients with ARDS: from widespread positive cells in the alveolar walls (arrows) (D), to almost no positive cells in the alveolar walls (E). Note that the latter picture is comparable to the finding in lung tissue of a patient who died with sepsis, but with normal lung architecture and without clinical ARDS (F).
precipitation with hyaline membranes, hemorrhage). Alterations indicating early exudative phase of ARDS were seen in only two patients (Figure 1A); the remaining fourteen patients showed evidence of early to late proliferative and fibrotic changes (Figure 1B-C).

Interestingly, the degree of active caspase-3 immunostaining in the lung tissues was highly variable between the ARDS patients, ranging from scarce positive lung cells to widespread staining in alveolar wall cells (Figure 1D-E). This finding was not related to inhomogeneous distribution of pathological changes, as we found comparable results in individual children of whom sections from multiple sites/lobes had been obtained. In injured lungs with extensive cellular infiltrates it may be difficult to reliably determine the cellular distribution of caspase-3 immunostaining. For this reason, we next performed double immunohistochemistry including an epithelial specific marker, (pan)cytokeratin. In addition, we used spectral imaging for quantification of the degree of co-localization of the signals (Figure 2A-B). Again, by this method there was a high variability between the

**Figure 2.** A, representative composite images of cytokeratin (blue) and active caspase-3 (red) double staining of lung tissue after unmixing of the individual color spectra, showing co-localization (yellow) (zoomed from 200x magnification images); B, original RedGreenBlue-bright field images (upper left) and composite images: without highlighted co-localization (upper right), active caspase-3 images (lower left) and cytokeratin images (lower right); C, percentage of cytokeratin (ck) positive staining co-localized with positive cleaved caspase-3 staining in alveolar tissue sections of ARDS patients with indirect lung injury (n=4) or direct lung injury (n=12); D, correlation between the percentage of cytokeratin (ck) positive staining co-localized with positive cleaved caspase-3 staining and age (r=0.5, p<0.05).
patients with ARDS: the median (range) percentage of positive cytokeratin pixels (surface area) co-localizing with positive active caspase-3 signal per image was 6.1% (1.0-18.1%). Importantly, we found a high correlation between visually counted double signal positive cells counted and the spectral imaging quantification ($r=0.9$, $p<0.01$, not shown).

No clear association was found between the percentage of cytokeratin staining co-localizing with active caspase-3 staining and cause of lung injury (indirect versus direct) (Figure 2C) or duration of mechanical ventilation ($r=0.3$, $p=\text{ns}$, not shown). For ARDS pathological stage a potential association could not be determined because almost all patients showed progressed alveolar injury. Interestingly, in our cohort, age was positively correlated with the degree of cytokeratin/active caspase-3 double positive staining (Figure 2D).

**DISCUSSION**

Classical (caspase-dependent) apoptosis is considered an important mechanism of epithelial injury in ARDS. In the present observational report we studied active caspase-3 immunostaining specifically in epithelial cells in lung tissues of children who died with ARDS. We introduce spectral imaging to quantify the extent of co-localization of active caspase-3 and the epithelial marker (pan)cytokeratin.

Our methods have the following advantages. First, the double immunostaining increases our insight into caspase activation specifically in lung epithelial cells, in this case alveolar epithelial cells as images contained alveolar structures only. Second, the use of spectral imaging overcomes problems with loss of color contrast often encountered in double immunostainings with co-localizing signals, and it quantifies the findings fast and likely more accurate over a large tissue area in comparison to semi-quantified data from visual counting by light microscopy. Finally, in the current analysis, spectral imaging helps to determine how much of the lung epithelium actually is involved in terms of activation of caspase-3; it can be used to calculate the percentage of epithelial surface area that shows active caspase-3 staining. Importantly, there was a high correlation between these quantified data based on double positive surface area (pixels) and the number of epithelial cells deemed positive for caspase-3 as visually counted.

The main finding of this study is a high variability in active caspase-3 staining in lung epithelium between the children with ARDS. We found a range including patients with almost no caspase-3 positive epithelial cells, comparable to a patient without ARDS, to patients with widespread positive caspase-3 staining in up to almost 20% of the epithelial surface area. Evidently, a number of different factors may have contributed to this variability. First, immunohistochemical staining of autopsy samples is limited to ‘snapshot’ analysis, and does not appreciate the dynamic character of apoptosis. Second, as is true in general in the ARDS population, the patients in our study were highly heterogeneous.
with respect to clinical disorders and underlying illnesses and their treatments. In this light, our study once more highlights the challenge encountered in ARDS research due to the non-specific clinical criteria on which the diagnosis is based. Although we found no association between the extent of caspase-3 activation and the above mentioned factors, we have to carefully interpret these findings because of the retrospective design and small number of patients in this study. Interestingly, despite this limitation, age positively correlated with the extent of caspase-3 immunostaining in lung epithelial cells. This finding is in line with emerging experimental and clinical studies that suggest age and lung development are important factors influencing pro-apoptotic pathways during lung injury, as well as the susceptibility and outcome of ARDS.\textsuperscript{8-13}
REFERENCES
