Smoke/Burn Injury-Induced Respiratory Failure Elicits Apoptosis in Ovine Lungs and Cultured Lung Cells, Ameliorated With Arteriovenous CO₂ Removal*

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Study objectives: The purpose of this study was to examine the effects of two supportive therapies, conventional mechanical ventilation (CMV) and arteriovenous CO₂ removal (AVCO₂R), during treatment of severe smoke/burn injury-induced ARDS.

Design: Sheep were exposed to a smoke/burn injury (lethal dose causing death in 40% of animals); lung tissue and blood was collected prior to injury (control), when an ARDS criterion was met (PaO₂/fraction of inspired oxygen ratio < 200), then after 72 h of either CMV (group 1) or AVCO₂R (group 2). Lung tissue was studied by standard histopathologic techniques; cultured lung cells were studied in media supplemented with serum from all four groups.

Measurements and results: In vivo assays demonstrate less apoptotic cell death, and in vitro assays show significantly greater (p < 0.05) cell survival in group 2 (AVCO₂R) than in group 1 (CMV) or baseline. Differential gene expression demonstrates significantly higher messenger RNA levels of proapoptotic and tumor necrosis factor (TNF)-α in cells incubated in baseline media. After exposure of cultured lung cells to conditioned media, protein expression assay of the culture medium revealed no TNF-α, TNF receptor (TNFR)-1, or TNFR-2, however, cultured cell lysate reveals elevated levels of TNF-α, TNFR-1 and caspase-3 in all groups; most occurred in cells incubated in baseline media (p < 0.05). HOECHST stain, DNA fragmentation, and caspase-3 cleavage show that AVCO₂R ameliorates apoptosis in this model.

Conclusions: This in vitro work specifically examines cell death in lung cells as a result of smoke/burn injury and effects of therapeutic interventions. Our in vivo studies temporally correlate the clinical pathology to that studied in these lung cells and show that both in vivo and in vitro cell death is predominantly apoptotic and is significantly reduced by AVCO₂R.

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Key words: ARDS; arteriovenous CO₂ removal; tumor necrosis factor

Abbreviations: ANOVA = analysis of variance; AVCO₂R = arteriovenous CO₂ removal; CMV = conventional mechanical ventilation; FIO₂ = fraction of inspired oxygen; LD₄₀ = lethal dose causing death in 40% of animals; PEEP = positive end-expiratory pressure; RR = respiratory rate; TNF = tumor necrosis factor; TNFR = tumor necrosis factor receptor; VT = tidal volume

The morbidity of a severe cutaneous burn injury is increased when accompanied by smoke inhalation,1–3 a stimulus for the inflammatory response.4,5 If severe enough, the inflammatory response may promote ARDS. The clinical definition of ARDS is rapidly progressive bilateral alveolar infiltrates with PaO₂/fraction of inspired oxygen (FIO₂) ratio < 200 and a pulmonary capillary wedge pressure < 18 torr, or echocardiographic evidence of normal left ventricular function (American-European Consensus Conference).6,7 Pathologically, endothelial and epi-
thelial injury with increased vascular permeability, interstitial pneumonitis, and extensive obliterator fibrosis with destruction of the normal lung architecture characterizes ARDS.8 Overall, ARDS affects 150,000 patients a year in the United States and results in approximately 30% mortality.9 ARDS associated with smoke inhalation and cutaneous burn (mortality of 50 to 80%) is a predominant cause of death in burn patients.2,10,11

ARDS results as a consequence of a systemic inflammatory response. Apoptosis, programmed cell death, is frequently increased during injury and inflammation in parenchymal tissues.12 However, little is known about apoptotic processes in the lung after burn injury. This self-destructive process is regulated by both external and internal signals. Pro-apoptotic signals may be transduced through cytokine receptors of the tumor necrosis factor (TNF) family,13 eicosanoids,4 reactive oxygen species, proteases,14 and mechanical stretch.15 This signaling results in the cleavage and activation of cell-death proteases called caspases (cysteinyl aspartate-specific proteinases)16; of these, caspase-3 is commonly recognized as the initiator of apoptosis. This results in several changes within the cell leading to chromatin condensation and enzymatic fragmentation of DNA and ultimately death. No individual assay can directly detect apoptosis, but by using a combination of methods (we selected HOECHST stain for condensed chromatin, DNA fragmentation, and caspase-3 cleavage) it can be characterized and quantitated.

ARDS is treated primarily with mechanical ventilation, which tends to normalize blood gases through manipulation of minute ventilation and oxygen concentration, often allowing the lungs to recover from the inciting incident.17 However, mechanical ventilation may cause hyperinflation with alveolar stretch that may aggravate the pulmonary inflammatory response, dramatically altering the mechanical properties of the lungs and resulting in reduction of static compliance and inadequate gas exchange.18,19 Alternative gas exchange strategies in burned patients focus on reducing this mechanical ventilator-induced lung injury, and include low tidal volume (Vt), high-frequency percussive ventilation, and extracorporeal gas exchange techniques.20–22

Using our established clinically relevant, large-animal model of smoke/burn-induced ARDS (lethal dose causing death in 40% of animals [LD40]),23 we have developed the technique of extracorporeal arteriovenous CO2 removal (AVCO2R) to reduce ventilator-induced lung injury.24–31 During AVCO2R, CO2 removal and O2 transfer are uncoupled, CO2 is transferred across the membrane gas exchanger, and O2 diffuses across the native lungs by low-frequency positive pressure ventilation,32 thus allowing for reduced Vt, peak inspiratory pressures, and minute ventilation during mechanical ventilation.30 This technique has been shown to improve survival in our ovine model of smoke/burn-induced respiratory failure.22 We have also demonstrated safety and proof of concept in humans with ARDS.19,20

The purpose of this study was to examine the effects of two supportive therapies, conventional mechanical ventilation (CMV) and AVCO2R, on lung tissue and cultured lung cells following the development of ARDS. Therefore, our hypothesis was that severe smoke/burn injury results in apoptotic lung cell death ameliorated by AVCO2R. In order to address the hypothesis, this study examined the following: (1) acute lung injury secondary to smoke/burn in sheep, (2) appearance of apoptosis in the lungs, and (3) the development of apoptosis as affected by different respiratory support strategies. A novel study design was applied in order to best isolate and study the observed pulmonary apoptosis. Sera from animals in each experimental group were used to supplement growth media for lung cells in culture, which could then be studied for the changes characteristic of apoptosis.

Materials and Methods

Experimental Design

Briefly, as shown in Figure 1 (preinjury sampling is control group), anesthetized sheep received an LD40 injury by a 40% third-degree cutaneous flame burn plus 36 breaths of cotton-smoke insufflation injury.21 The animal was then supported by a mechanical ventilator with settings adjusted to maintain an arterial pH between 7.35 and 7.45, PaO2 > 60 mm Hg, and PaCO2 < 40 mm Hg.22 If the animal achieved ARDS criteria ([PaO2/FIO2 ratio < 200] at sampling time point is baseline group), it was randomly assigned to 72 h of support by either CMV (group 1) or AVCO2R (group 2).22–24 For in vivo experiments, sections of ovine lung from each group were studied by standard histopathologic techniques. For in vitro cell culture and biochemical experiments, serum was collected from these four groups, and its effect on lung cells grown in culture was studied by cell culture and biochemical assays.33

In Vivo Model Development

Adult free-range sheep (3 to 4 years old; 35 to 45 kg) were administered sufficient ketamine (12.5 mg/kg IM, ± 5.0 mg/kg IV) to achieve initial sedation for intubation, induction continues with 4% halothane via an anesthesia ventilator (Ohmeda 7000; BOC Health Care; Liberty Corner, NJ).21 Surgical anesthesia was maintained with 1 to 2.5% halothane titrated to a heart rate of 75 to 120 beats/min. A 20% total body surface area, third-degree cutaneous flame burn was administered to each flank (40% total), with 36 breaths of cotton-smoke insufflation delivered between the two burn injuries. Following the injury procedure, each sheep was recovered, transferred to the ICU, and placed on mechanical ventilation (Servo 900C; Siemens-Elema; Solna, Sweden) with hemodynamic monitoring. Initial postinjury ventilator settings
were as follows: respiratory rate (RR), 25 to 30 breaths/min; Vt, 15 mL/kg; FiO₂, 1.0; and positive end-expiratory pressure (PEEP), 5 cm H₂O. When animals met the criteria for ARDS (PaO₂/FiO₂ ratio < 200), generally within 40 to 48 h of injury, they were either connected to CMV or returned to the operating room for placement of AVCO₂R.

AVCO₂R animals underwent cannulation of the left carotid artery (Percutaneous 10 Fr. TF018LH; Research Medical; Midvale, UT) and left jugular vein (Percutaneous 14 Fr. TF022L; Research Medical) via sterile cutdown. Immediately prior to removal, minute volume was reduced in a stepwise fashion (20% reduction) to reduce the positive end-expiratory pressure casts. Airway suctioning and lavage were performed every 4 to 6 h to remove proteinaceous bronchial casts.

**Cell Culture**

The human lung cell line—BEAS2-B (immortalized bronchial epithelial)—was used in these experiments and was cultured in humidified 95% air, 5% CO₂ incubators at 37°C (RPMI 1640; BioWhittaker; Walkerville, MD) 90%, fetal bovine serum 10%. Cells were grown on 100-mm plates to 70% confluency before treatment. All experiments were done in triplicate with at least three samples per data point.

**Conditioned Media**

Ovine blood collected from at least three sheep in each group was separated into serum and cellular portions by centrifugation (10 min, 1,000 revolutions per minute, 22°C). This serum (10%) was then substituted for the fetal bovine serum in standard media for this study. The following four groups were developed: (1) preinjured sheep (control), (2) sheep after they met ARDS criteria from smoke/burn injury (baseline), (3) sheep after 72 h of CMV for ARDS (group 1), and (4) sheep after 72 h of AVCO₂R support for ARDS (group 2).

**Preliminary Experiments**

Because BEAS2-B cells are usually cultured in media containing 10% fetal bovine serum, we first determined the effect of substituting ovine serum for fetal bovine serum. Therefore, the 96-h growth curve and HOECHST assay was performed on BEAS2-B cells grown in media containing either 10% fetal bovine serum or 10% ovine serum.

**Mass Culture Assay**

Five 12-well tissue culture plates were plated with 10⁵ cells in standard fetal bovine media. Twenty-four hours later, the media was changed to one of the following per plate: control ovine media, baseline ovine media, group 1 (CMV) ovine media, and group 2 (AVCO₂R) ovine media. Cells were harvested using trypsin, and the total number of cells per well was determined (Coulter Model A or Z; Coulter Electronics; Hialeah, FL) count at the following time points: before media change, and at 24 h, 48 h, 72 h, and 96 h later. This gave a triplicate count for each time point after the media change.

**Apoptosis Index**

HOECHST 33258 dye (B2883; Sigma Chemical; St. Louis, MO), an ultraviolet light-excitable dye that demonstrates increased fluorescence when bound to the condensed chromatin of apoptotic cells, was used to quantify apoptotic cells in both lung parenchymal tissue and cell culture. In tissue culture, once cells had reached 70% confluency in normal fetal bovine sera, the media was changed to study media: either control, baseline, group 1 (CMV)-, or group 2 (AVCO₂R)-conditioned media.
48 h, the cells were fixed with cold 95% ethanol and stained with 1 μg/mL HOECHST dye. Randomly selected sections of ovine lungs were fixed with 95% ethanol and stained for apoptosis (Hoechst 33258 dye). The plates of cells and lung sections were then observed under a Nikon Diaphot 300 fluorescence microscope (Nikon USA; Garden City, NY) and results expressed as the apoptotic index (number of apoptotic cells/total number of cells in a field).

**Histopathology**

Randomly selected sections of ovine lungs were collected from baseline, group 1 (CMV) or group 2 (AVCO₂R) animals; these sections were fixed in neutral-buffered formalin. Following routine processing, sections were stained with hematoxylin-eosin (photomicroscopy at 100×) and studied in a blinded manner by a pathologist (P.J.B.).

**Trypan Blue Assay**

Trypan blue dye, excluded by the intact plasmalemma of live cells, was used to indicate cell viability.⁴⁴ Cells were grown to 70% confluence in 75-mm plates (Culture Dish; Becton Dickinson; Franklin Lakes, NJ) in normal fetal bovine media, which was then changed to group 1 (CMV) or group 2 (AVCO₂R) media. After 24 h, 100 μL of a 0.4% solution of trypan blue was added to each plate and allowed to stand for no more than 5 min. The percentage dead (determined by dividing the number of dead cells by total number of cells) was recorded.

**RNA Isolation and Purification**

BEAS₂⁻B cells were cultured in either baseline, group 1 (CMV)- or group 2 (AVCO₂R)-conditioned media in T175 flasks for 24 h, cells were lysed and RNA isolated, which was then deoxyribonuclease treated (Atlas Pur Total RNA Labeling System; Clontech Laboratories; Palo Alto, CA). RNA yield and purity were assessed using a Beckman DU-64 Spectrophotometer (Beckman Instruments; Fullerton, CA).

**Probe Synthesis**

Purified total RNA was converted into ³²P-labeled, first-strand complementary DNA using Moloney murine leukemia virus reverse transcriptase (1 mL per reaction). In order to purify labeled complementary DNA probes from excess labeled nucleotides, the Atlas NucleoSpin Extraction Kit (Clontech Laboratories) was used.

**Hybridization**

Each Atlas Human Apoptosis Array (Clontech Laboratories) was placed into hybridization bottles, prehybridized, and mixed with 0.8-g heat-denatured, sheared salmon testes DNA (10 mg/mL; Sigma D7566; Sigma Chemical) at 68°C. Following this incubation, group 1 (CMV), group 2 (AVCO₂R), and baseline hybridization-ready complementary DNA probe samples were added directly to the membranes already incubating in prehybridization solution. Differential expression analysis was carried out with AtlasImage 1.0 for Windows software (Clontech Laboratories).

**Protein Expression**

Briefly, after 48 h of exposure, both cell lysate and culture media were harvested at all time points, and assayed with Bradford assay for protein quantification.³⁵ Each lane was loaded with 50 μg of either cell lysate or culture medium. Primary antibodies used included the following: anti-TNF receptor (TNFR)-1 (1:100, N-20, SC-1067) and anti-TNF-α (1:100, L-19, SC-135) [Santa Cruz Biotechnology; Santa Cruz, CA]. Secondary antibody used was anti-goat IgG-horseradish peroxidase (1:1000, SC-2020). Caspase antibody was anti-caspase-3 (1:100, AAF-113; StressGen; Victoria, BC, Canada). Actin (anti-actin sc-1615; Santa Cruz Biotechnology) was utilized as a Western blot loading control. Positive controls for caspases were either heat-shocked HeLa cell lysate (LYC-HL101; StressGen), or 24-h incubation of cells with camptothecin (known to induce apoptosis). Secondary antibody was goat anti-rabbit IgG standard-horseradish peroxidase (1:1000, SAB-300, StressGen Corporation). The membranes were incubated for 2 h, bands were visualized, a densitometric analysis was performed by UNSCAN-It software (version 5–1; Silk Scientific; Orem, UT), and data are reported and graphed as the average relative difference per pixel over a fixed area from at least three runs (mean ± SD).

**DNA Laddering**

Cells were counted on a Coulter Counter and prepared for DNA ladder assay using the Quick Apoptotic DNA Ladder Kit (BioVision; San Diego, CA). Positive control was produced using 1 μL camptothecin in 10 mL of media added to cells in bovine sera; negative control was cells in bovine media. Study groups were control, baseline, group 1 (CMV), and group 2 (AVCO₂R). Cells were harvested at 8 h and lysed with Tris boric acid ethylenediamine tetra-acetic acid lysis buffer. DNA was degraded with ribonuclease and protein with proteases. DNA was precipitated with ammonium acetate and absolute alcohol. After air-drying, DNA was dissolved in a suspension buffer, loaded into a 1.5% agarose gel, and run on agarose electrophoresis.

**Statistical Analysis**

Values are expressed as the mean ± SEM. When comparing cell numbers before (baseline) and after exposure to test media (group 1 [CMV] or group 2 [AVCO₂R]), the paired t test was used; otherwise, the repeated-measures analysis of variance (ANOVA) was used. The level of significance was set at p < 0.05.

**Results**

**Preliminary Studies**

A 96-h growth curve comparison indicates that the BEAS₂⁻B cells have a reduced number of cells when grown in ovine sera (data not shown). However, a HOECHST stain assay for condensed chromatin (a hallmark of apoptosis) indicates that there is no increase in cell death when grown in this media; therefore, the reduced number of cells is the result of a slower growth rate.

Ratio of PaO₂/FiO₂ (Fig 2) compared between groups over time of experiment reveals that 48 h after injury entering criterion was met (AVCO₂R and CMV, PaO₂/FiO₂ ratio 194.8 ± 26). After 36 h of support, PaO₂/FiO₂ ratio for AVCO₂R animals was > 200, whereas the CMV animals show no improvement (ANOVA, p < 0.05).
Mass Culture Assay

When the human lung cells were grown in media containing the serum from the control, baseline, and the two treatment groups (group 1 [CMV] and group 2 [AVCO2R]), the following growth rates were calculated (+ indicates proliferation, − indicates decrease). Baseline (slope $\pm 4,811$ cells per day), group 1 (slope $\pm 7,880$ cells per day), and group 2 (slope $\pm 6,354$ cells per day) display significantly reduced number of cells over 96 h compared to control (slope $\pm 33,333$ cells per day). Figure 3 shows 96-h growth curve for all groups. The mitotic rate or doubling time was similar (t test, p $\geq 0.05$) between the control group (0 to 24 h) and group 2 (72 to 96 h) when their total cell population numbers were similar. The cells were retained in the “conditioned” media for the duration of this experiment; apoptosis assessments were employed to determine if the decreased cellular survival was due to apoptosis.

Apoptosis Assay

The first phase (Fig 4, top, A) of this analysis compared the apoptotic index data from HOECHST staining in lung parenchyma between animals in group 1 (CMV) and group 2 (AVCO2R) after 72 h of either support regimen (Fig 4, bottom, B). The lungs of animals in group 1 have significantly more apoptotic cells present: group 1, 50.7 $\pm 3.2$ cells/100 cells; vs group 2, 25.0 $\pm 8.0$ cells/100 cells (p $\leq 0.05$).

Histopathology

Histopathologic sections of lungs from sheep at the time when they reached ARDS criteria (baseline) showed severe, organizing interstitial pneumonitis with extensive fibrosis obliterating the normal alveolar architecture (Fig 5, top, A). When CMV was instituted following injury, the degree of interstitial organization (Fig 5, center, B) and fibrosis appeared clearly diminished. The alveolar walls were partially or focally thickened by fibrosis, but the majority showed only a mild degree of widening (Fig 5, center, B). In group 2 (AVCO2R) sheep, the alveolar architecture was essentially normal in large areas of lung parenchyma, with only rare areas of mild thickening of alveolar walls and resolving, interstitial pneumonitis (Fig 5, bottom, C).

Trypan Blue Assay

Significantly more trypan blue positive (dead) cells were detected in group 1 (CMV) than in controls ($10^3$ cells; and group 2, $15.0 \pm 1/100$ cells) than control ($5.2 \pm 1.1/100$ cells; ANOVA, p $\leq 0.05$). Group 2 had significantly fewer apoptotic cells than baseline.

The second phase of this analysis compared the number of apoptotic cells from HOECHST staining in lung parenchyma between animals in group 1 (CMV) and group 2 (AVCO2R) after 72 h of either support regimen (Fig 4, bottom, B). The lungs of animals in group 1 have significantly more apoptotic cells present: group 1, 50.7 $\pm 3.2$ cells/100 cells; vs group 2, 25.0 $\pm 8.0$ cells/100 cells (p $\leq 0.05$).

Histopathology

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Trypan Blue Assay

Significantly more trypan blue positive (dead) cells were detected in group 1 (CMV) than in control.
Differential Expression Analysis

Comparison of both group 1 (CMV) and group 2 (AVCO₂R) to baseline and to each other revealed differences in human lung cell gene expression. TNF-related genes and other proapoptotic genes were expressed higher in group 1 when compared to group 2. Table 1 compares all genes with a twofold or greater difference in expression between the two groups or when compared to baseline. Proapoptotic signals include TNFR-1, TNFR-1-associated protein, BAK, caspase-3, PDCD2, and Fas-activated serine/threonine kinase. Additionally, group 1 demonstrated increased expression of three antiapoptotic genes compared to the group 2: survivin, bcl-2 binding anthanogene, and c-Jun.

Protein Expression

Western blot of ovine sera for TNF-α reveals no detectable TNF-α in culture media from control, group 1 (CMV), group 2 (AVCO₂R), and baseline (Fig 6, top, A). Additionally, lung tissue-culture cell lysate was probed for TNF-α; results show that TNF-α was detected in all human lung cell lysate samples with the greatest amount present in group 1 (156 ± 9 U; Fig 6, bottom, B). This finding was found to be the same for TNFR-1 (91 ± 8 U, mean ± SD).

Caspase-3

Detection of apoptosis-related caspases was performed using Western blot on human lung culture cell lysate exposed to sera for 8 h from control and all study groups. Figure 7, top, A, shows the presence of both uncleaved and cleaved caspase-3. Injury groups of baseline, group 1 (CMV), and group 2 (AVCO₂R) had cleaved caspase-3, the active form of the enzyme. In the injury groups, less uncleaved caspase-3 was evident in group 1 compared to group 2 cells (Fig 7, bottom, B).
DNA Fragmentation Assay

Figure 8 shows the results for DNA fragmentation for 8 h after introduction of conditioned media to cells in culture. Results showed DNA fragmentation clearly evident in all injury samples. DNA laddering showed significantly more fragmentation in baseline and group 1 (CMV) and group 2 (AVCO2R) [p < 0.05] than in control.

**DISCUSSION**

We studied the effect of our LD_{40} smoke/burn injury model of ARDS on the lungs of adult sheep, and correlated these effects with those seen when growing cells in media supplemented with serum from these animals. This novel study design relates events in vivo to those in vitro. Our initial, in vivo studies included characterization of lung morphology and quantification of apoptosis from histopathologic samples of lung parenchyma of these sheep. Both AVCO2R and mechanical ventilation continued for 72 h after ARDS, and both showed decreased pathology from the time at which an ARDS criterion was met, with AVCO2R group showing greater normalization than the CMV group. Our in vitro studies included characterization of cellular morphology and quantification of apoptosis and additionally studied known molecular inducers of apoptosis at both messenger RNA and protein levels. Data revealed that sera from animals in the ARDS baseline and group 1 (CMV) treatment groups resulted in more apoptosis in cultured lung cells than sera from group 2 (AVCO2R)-treated animals. Complementary DNA expression analysis of these cultured cells verified by Western blot revealed increased levels of several proapoptotic (caspase) and proinflammatory (TNF-related) genes, thus supporting apoptosis as a possible mechanism for the increased cell death.

In this large animal/cell culture model, the smoke insufflation and cutaneous burn injuries occur simultaneously, and create an imbroglio where their deleterious effects are inseparable. The end result is respiratory failure, which is followed by death in 40% of the animals. Tissue damage caused by heat results in an immune response leading to the upregulation of cytokines that can potentially further exacerbate lung injury.5 Smoke particles can also cause cellular dysfunction, cell death, and stimulation of the inflammatory response.5,36 Mechanical ventilation has been shown to predispose patients to the development of systemic inflammatory response that leads to local tissue injury, multiple organ dysfunction, and

**Table 1—Comparison of Gene Expression**

<table>
<thead>
<tr>
<th>Gene</th>
<th>CMV</th>
<th>AVCO2R</th>
<th>Fold Difference</th>
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<tr>
<td>BAK</td>
<td>↑ 1.41</td>
<td>↓ 2.46</td>
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<td>TNF-α</td>
<td>↑ 2.60</td>
<td>↑ 3.50</td>
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<td>ERK6</td>
<td>↑ 3.10</td>
<td>3.10</td>
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<tr>
<td>c-Jun</td>
<td>↑ 13.4</td>
<td>↑ 1.86</td>
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<td>Caspase-3</td>
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<td>TNF-R1</td>
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<td>DR51</td>
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<td>↑ 4.6</td>
<td>6.67</td>
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<td>IGFBP3</td>
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<td>5.62</td>
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<td>FAST kinase</td>
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<tr>
<td>Survivin</td>
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<tr>
<td>Phospholipase A_{2}</td>
<td>↑ 5.3</td>
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*Comparisons between Group 1 (CMV) and Group 2 (AVCO2R) to control greater than twofold difference.
†Cytotoxic TRAIL receptor 2.
The result in these animals (LD40) was an increased presence of apoptotic cells in the respiratory tissue as well as an almost total obliteration of normal architecture (Figs 4, 5). The effects on lung cells in culture showed an increase in the number of apoptotic cells as documented by DNA fragmentation (Fig 8) and Hoechst staining (Fig 4, top, A). Additionally, analysis at the molecular level revealed no TNF-α present in the cell culture medium; however, when cells were grown in this media, TNF-α messenger RNA and TNF-α protein as well as caspases-3 were upregulated. The result in 40% of animals is ARDS.

In sheep that met ARDS criteria and were randomized to CMV (group 1), there was a significant decrease in number of apoptotic cells and an observed improvement in lung architecture by histopathology. The effects of serum from group 1 on cultured lung cells showed a decrease in the amount of apoptotic cells present; however, the remaining number of apoptotic cells still greatly exceeded that of cells grown in sera from uninjured sheep. The results at the molecular level revealed the following when compared to uninjured: an increase in many proapoptotic and TNF family messenger RNAs, no TNF-α detectable in the ovine serum by protein expression analysis, increased expression of both TNF-α and TNFR-1 and caspase 3 in cultured cell lysate, as well as DNA fragmentation. This comparison reveals that CMV after ARDS criteria are met results in a decrease in pathophysiology. Improv-
ment from baseline occurs at the tissue and cellular levels for proapoptotic signals, and offers molecular evidence for apoptosis as a major contributor for the increase in cell death. This indicates that the therapy has decreased, but did not eliminate the putative apoptosis mediator.

For the sheep that met ARDS criteria and were randomized to AVCO2R (group 2), the lung architecture after 72 h of support was essentially normal indicating near total recovery from the initial injury (Fig 5, bottom, C). Additionally, reduced apoptosis compared to baseline was noted, although the number of apoptotic cells still significantly exceeded the uninjured control. At the cellular level, the growth curve (Fig 3) and presence of apoptosis (Fig 4) in group 2 were also greatly improved from baseline (ARDS). At the molecular level, gene array results indicated increased levels of several proapoptotic factors from uninjured control animals, especially TNF-related factors. Again, no TNF-\(\alpha\) was detected in ovine sera; however, both TNF-\(\alpha\) and TNFR-1 were found in cultured human lung cells incubated with this serum. Uncleaved caspase-3 protein expression levels were increased compared to baseline in this group. Seventy-two hours of AVCO2R after ARDS criterion was met resulted in improvement from baseline with both morphologic and molecular evidence for apoptosis as a major contributor for the increase in cell death, and indicates that the stimulus for apoptosis has been either removed or greatly diminished.

The decrease in the stimulus for and number of apoptotic cells in AVCO2R compared to CMV could be the result of several factors. With the institution of AVCO2R, the animal is provided “lung rest,” which may remove or diminish some of the elements of the “triple-hit” model, especially the effects of repeated stretching of the lungs during respiration. Bidani et al\(^6\) reported that smoke and burn injury resulted in a “priming” of alveolar macrophages for lipopolysaccharide-induced release of TNF-\(\alpha\), and Peter et al\(^{39}\) showed an enhanced TNF-\(\alpha\) activity in burn injury. Hales et al\(^4\) were unable to demonstrate a role for TNF-\(\alpha\) in sheep after smoke and burn injury; our study extends their findings and takes place much later, after the application of supportive therapy. We are in agreement with Clancy et al,\(^40\) who did not document elevated TNF-\(\alpha\) levels when they examined whole-organ responses and speculate that elevated systemic levels of TNF-\(\alpha\) likely represent a late manifestation of the inflammatory response. In agreement with others is the fact that we were unable to detect TNF-\(\alpha\) in ovine sera,\(^{41}\) although we did detect the result of its effect on cells grown in the serum. This implies that the putative mediator may be something other than TNF-\(\alpha\).

Repeated mechanical stress has been shown to stimulate the stress-activated protein kinase pathway, resulting in an upregulation of proapoptotic cytokines such as TNF family of proteins.\(^{15,42}\) Activation of members of this family may induce apoptosis through two pathways: the extrinsic pathway\(^43\) and the intrinsic pathway.\(^{44}\) Both pathways converge at caspase-3, the effector for apoptosis. Our DNA fragmentation and HOECHST experiments clearly reveal that apoptosis results from the injury, and that it is greatly reduced with CMV and AVCO2R. Our data also make clear that cleaved caspase-3 and cytosolic TNF-\(\alpha\) are significantly increased in the injured cell populations implying apoptosis, and that this increase is attenuated with both interventions.

In conclusion, sheep with smoke/burn-induced severe respiratory failure exhibit significant apoptosis of lung cells in vivo, and sera from these sheep induce apoptosis in cultured human lung cells in vitro. The fact that both the lungs of the sheep and human lung cells in culture displayed apoptosis implies that this may be an excellent model combination to study the cellular and molecular effects of the initial injury and subsequent interventions. Our study of the role of TNF-\(\alpha\) takes place only in cell culture where human lung cells are reacting to the “conditioned media,” and may not be representative of an end-organ response. Clearly, mediator(s) are present in all three experimental media that are not present in the control media, which induce apoptosis in a caspase-3–dependent manner. We have shown increased messenger RNA expression of key apoptotic mediators (such as TNFR-1, caspase 3, DR5, TNFR-1–associated protein) following smoke/burn injury, but still have not identified the inciting agent. Although we were unable to detect any increase in TNF-\(\alpha\) or TNFR-1 in ovine sera, we did observe an increase in TNF-\(\alpha\) in cells grown in these sera, thus suggesting that some other agent may be present in the serum that induces TNF-\(\alpha\) expression in these cultured cells. TNF-\(\alpha\) has also been shown to play a protective role in special circumstances\(^{45}\); in light of the fact that we found increased messenger RNA for several antiapoptotic proteins such as survivin and c-Jun, this needs to be determined. The stress-activated protein-kinase cascade or some other proapoptotic stimulus may activate apoptosis that stimulates TNF-\(\alpha\) expression in an autocrine manner. Future work will focus on corroborating these results: molecular studies seeking the identity of this “inciting agent” and mechanistic studies aimed to explain the improvements in outcomes seen with AVCO2R and other mechanical ventilation strategies. Early application of AVCO2R or other strategies to reduce lung stretch may decrease permanent/irreversible lung damage during a smoke/burn injury.
and possibly in other forms of acute lung injury requiring mechanical ventilation.

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