Targeting ARDS
Molecular Biology: Events in the Cell

Clifford S. Deutschman, MS, MD, FCCM
Professor of Anesthesiology and Critical Care
Perelman School of Medicine at the University of Pennsylvania
Targeting ARDS
Molecular Biology: Events in the Cell
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Molecular Biology: Events in the Cell

Clifford S. Deutschman, MS, MD, FCCM
President-Elect
Society of Critical Care Medicine
(one of those “be careful what you wish for” jobs)
For example, I am required to present, and you are required to endure.
Disclosures
Disclosures

– Royalties from Elsevier for our Critical Care book
  – to which a number of people at this meeting contributed
    • and which is a really, really good book
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- No conflicts
Major Conflict
Major Conflict

Penn Undergrad
Grad School in New York
Dickinson Undergrad
Major Conflict

Un-Conflicted but Broke
(and, as an SCCM officer, sworn to stay that way)
Collaborators
(People who really did the work)
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– Yoram G. Weiss, MD
  – Chair, Dept of Anesthesia and Critical Care Medicine, Hadassah/Hebrew University Medical School
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  • Nichelle Raj, MS
  • Melanie Lyons, MSN

– Laurie Kilpatrick, PhD
  – Associate Professor, Physiology and Director, Center for Inflammation, Translational and Clinical Lung Research, Sol Sherry Thrombosis Research Center, Temple University, Philadelphia, PA
Pathological Processes = Therapeutic Targets
– “Exuberant” Inflammation
– Neutrophil Accumulation
– “Exuberant” Inflammation
  – Neutrophil Accumulation
– Type I Cell Loss
  – Apoptosis
– “Exuberant” Inflammation
  – Neutrophil Accumulation
– Type I Cell Loss
  – Apoptosis
– Type II Cell Hyperproliferation
  – Cell replication
Pathological Processes

= 

Therapeutic Targets

– “Exuberant” Inflammation
  – Neutrophil Accumulation
– Type I Cell Loss
  – Apoptosis
– Type II Cell Hyper-Proliferation
  – Cell replication
Model – ARDS Secondary to Septic Peritonitis – Double puncture CLP in male Sprague–Dawley Rats
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YG Weiss et al, Anesthesiol., 2001
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“Exuberant” Inflammation

Neutrophils/Low Powered Field

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<th>Intervention</th>
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<tbody>
<tr>
<td>No Operation</td>
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<tr>
<td>Sham Operation (SO)</td>
<td>17 +/- 6</td>
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<tr>
<td>2CLP</td>
<td>914 +/- 156*</td>
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## “Exuberant” Inflammation


### Neutrophils/Low Powered Field

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Loss of Type I Cells

Aquaporin-1 Staining

Loss of Type I Cells

Aquaporin-1 Staining

$T_0$  CLP

Over-Proliferation of Type II Cells

Pro-SPC Staining

Over-Proliferation of Type II Cells

Pro-SPC Staining

Over-Proliferation of Type II Cells

Pro-SPC Staining

Therapeutic Targets
Therapeutic Targets – ✔
Therapeutic Agents – ✔
Therapeutic Agents – ✓

Something like…..

Heat Shock Proteins
What Are Heat Shock Proteins?
What Are Heat Shock Proteins?

– Part of the phylogenetically conserved Heat Shock Response
What Are Heat Shock Proteins?
– Part of the phylogenetically conserved Heat Shock Response
What Are Heat Shock Proteins?
– Part of the phylogenetically conserved Heat Shock Response

Lowest Life Form – Amoebas

Highest Life Form – Intensivist
What Are Heat Shock Proteins?
– Part of the phylogenetically conserved Heat Shock Response

(Not Tina Fey)
What Are Heat Shock Proteins?
What Are Heat Shock Proteins?

- Heat Shock Response
  - Protein expression evoked by a myriad of cellular stresses.
    - Heat/Heavy Metal Intoxication
    - Hypoxia, Shock, Endotoxemia
    - Ischemia/Reperfusion
  - Rapid Transcription, Long Protein Half-Life.
    - Allows Cellular Tolerance of Otherwise Lethal Insults.
- Key protein – HSP–70
What Does HSP–70 Do?

– Conserves, Restores, Alters Protein Structure by Interacting With Proteins and Polypeptides.

– Regulation of Translocation, Import and Folding
  » Clarke, Cell Stress Chaperones, 3:228–236, 1998

– Prevention of Aggregation and Restoration of Function of Denatured Proteins
Why use HSP–70 as our Therapeutic Agent?
Why use HSP–70 as our Therapeutic Agent?

– “Exuberant” Inflammation

– Attenuation of Inflammatory Mediator Expression


Why use HSP–70 as our Therapeutic Agent?

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– Type I Cell Loss
  – Inhibition of Apoptosis
    » Beere and Green, Trends in Cell Biology 11:6–10, 2001

– Type II Cell Hyper–proliferation
  – Interferes with cell replication

Use HSP–70 to explore/reverse
Model – 2CLP

– AdHSP administered into trachea at the time of 2CLP
  – Attenuated adenoviral vector expressing porcine HSP–70
    • HSP–70 mRNA from Antonio DeMaio
The Model – 2CLP + AdHSP

The Model – 2CLP + AdHSP

The Model – 2CLP + AdHSP

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The Model – 2CLP + AdHSP

**“Exuberant” Inflammation**


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# Exuberant Inflammation


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Loss of Type I Cells

Aquaporin-1 Staining

20X

T₀  2CLP–PBS  2CLP–HSP

40X

Loss of Type I Cells

Aquaporin-1 Staining

T₀  2CLP–PBS  2CLP–HSP

20X

40X

Loss of Type I Cells

Enhanced HSP–70 Expression with AdHSP Attenuates Proliferation of Type II Alveolar Cells

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Enhanced HSP–70 Expression with AdHSP Attenuates Proliferation of Type II Alveolar Cells

Pro-SPC Staining

20X

40X

So...
So...

- Administration of AdHSP to animals at the time of 2CLP
  - Improved histology
  - Decreased neutrophil accumulation
  - Preserved Type I cells
  - Impaired proliferation of Type II cells

-Mechanisms
Inhibition of NF-κB by Hsp70

Inhibition of NF–κB by Hsp70


300-350kD
Inhibition of NF-κB by Hsp70


- IκB Kinase (IKK) Activity
  - In inflammation, the active subunit is IKKβ
  - IKKβ is activated by phosphorylation
  - ELKS “presents” p65/p50/IκB to IKKβ
  - IKKβ phosphorylates IκB
  - IκB is targeted for ubiquitination and proteolysis
  - NF-κB translocates to nucleus, initiates transcription

26S Proteosome

300-350kD

p50

p65

IKKα

IKKβ

NEMO

ELKS
Inhibition of NF-$\kappa$B by Hsp70

Multimers are more active than monomers
Inhibition of NF-κB by Hsp70


Multimers are more active than monomers
Inhibition of Inflammation

NF-κB

NF-κB Responsive Gene

TNFR1

Ubiquitin Ligase

IKKγ
IKKα
IKKβ

p65
IkBa

p50

26S Proteosome

NF-κB Responsive Gene
Inhibition of Inflammation

NF-κB

TNF

TNFR1

IKKγ

IKKα

IKKβ

NF-κB Responsive Gene

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26S Proteosome

p65

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p50
Inhibition of Inflammation

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NF-κB

TNF

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IKKγ
IKKα
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IκBa P

Ubiquitin Ligase

26S Proteosome

NF-κB Responsive Gene
Inhibition of Inflammation

NF-κB

TNF

TNFR1

IKKα
IKKβ
IKKγ

p65
p50
IkBα

Ubiquitin Ligase

26S Proteosome

NF-κB Responsive Gene
Inhibition of Inflammation

NF-κB

Ubiquitin Ligase

26S Proteosome

Pre-mRNA
For TNF, IL-1, IL-6, IL-8

NF-κB Responsive Gene

TNF

IKKγ
IKKα
IKKβ

TNFR1

p65
p50

p65

p50

For TNF, IL-1, IL-6, IL-8
Inhibition of NF-κB by Hsp70

AdHSP Blocks… NF–κB DNA Binding

- TNF
- TNFR1
- IKKγ
- IKKα
- IKKβ
- p65
- p50
- IkBα
- Ubiquitin Ligase
- 26S Proteosome
- Pre-mRNA
- AdHSP NF–κB Responsive Gene
AdHSP Blocks…
NF-κB Nuclear Translocation

TNF
TNFR1

IKKγ
IKKα
IKKβ

p65
p50
IkBa

Ubiquitin Ligase

26S Proteosome

AdHSP
Pre-mRNA

AdHSP Blocks NF-κB Responsive Gene
AdHSP Blocks... 

**IκBα** Phosphorylation

- **TNF**
- **TNFR1**
- **IKKγ**
- **IKKα**
- **IKKβ**
- **p65**
- **p50**
- **IkBα**
- **Ubiquitin Ligase**
- **26S Proteosome**
- **AdHSP**
- **Pre-mRNA**
- **NF-κB Responsive Gene**
AdHSP Blocks…
IKKα Phosphorylation
HSP–70 either prevents IKK/IκB/NF-κB complex formation or stabilizes monomers and dimers.
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HSP-70 either prevents IKK/Ikβ/NF-κB complex formation or stabilizes monomers and dimers.
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HSP–70 either prevents IKK/IκB/NF–κB complex formation or stabilizes monomers and dimers. HSP–70 can bind to any of the individual components of the IKK/IκB/NF–κB complex but seems to have a preference for NEMO or p65.
Enhanced HSP–70 Expression with AdHSP Attenuates Apoptosis of Type I Alveolar Cells

Aschkenasy G, Bromberg Z, Raj N, Deutschman CS, Weiss YG. Plos Med In press
Enhanced HSP–70 Expression with AdHSP Attenuates Apoptosis of Type I Alveolar Cells


TUNEL Staining

- T0
- 2CLPPBS
- 2CLPAdHSP
- Positive control
- 2CLPPBS
- 2CLPAdHSP
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TUNEL Staining

Positive control-

T0

2CLPPBS

2CLPAdHSP

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Diagram:
- Apoptotic Stimuli
- Cytochrome c
- Mitochondrion
- Apaf-1
- dATP/ATP
- Apoptosome
- Procaspase-9
- AdHSP
- Procaspase-3
- Caspase-3
- Pro-caspase-8
- Caspase-8
- Apoptotic
- “Death”
Enhanced HSP–70 Expression with AdHSP Attenuates Apoptosis of Type I Alveolar Cells

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Enhanced HSP–70 Expression with AdHSP Attenuates Apoptosis of Type I Alveolar Cells

**Gel Filtration Chromatography**

**IP Caspase-9**

- 1500-900 kDa
- 600-400 kDa
- 400-300 kDa
- 200-100 kDa

- Apaf-1
- Caspase-9
- Caspase-8
- Caspase-3
- Hsp70

2CLPBB, 2CLPAdHSP, 2CLPBB, 2CLPAdHSP, 2CLPBB, 2CLPAdHSP
Enhanced HSP–70 Expression with AdHSP Attenuates Apoptosis of Type I Alveolar Cells

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Aschkenasy G, Bromberg Z, Raj N, Deutschman CS, Weiss YG. Plos Med In press

AdHSP impairs caspase – 8 and – 9 activation and activity, impairs interaction between caspase – 9 and Apaf, blocks translocation of activated Caspase – 3 into the nucleus and stabilizes lower molecular weight complexes containing Caspases –8, –9 and – 3
Enhanced HSP–70 Expression with AdHSP Attenuates Proliferation of Type II Alveolar Cells

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E2F1 Target Genes e.g., DNA Polα
Enhanced HSP–70 Expression with AdHSP Attenuates Proliferation of Type II Alveolar Cells

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Enhanced HSP–70 Expression with AdHSP Attenuates Proliferation of Type II Alveolar Cells

E2F1 Target Genes e.g., DNAPo1α.
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- AdHSP stabilizes $E_2F - Rb$
Enhanced Hsp70 Expression with AdHSP Attenuates Proliferation of Type II Alveolar Cells


- AdHSP stabilizes E$_2$F – Rb

![Image of a diagram showing the stabilization of E$_2$F and Rb with AdHSP]
Enhanced Hsp70 Expression with AdHSP Attenuates Proliferation of Type II Alveolar Cells

- AdHSP stabilizes E2F – Rb
AdHSP attenuates 2CLP–induced pulmonary hyper-proliferation in part by stabilizing E2F–pRb Complexes.
Summary
Summary

Summary


– This occurs, in part, because of
  – Limited inflammation
  – Attenuated type I cell apoptosis
  – Decreased type II cell hyperproliferation
Summary
Summary

- The effects appear to involve interaction of HSP-70 with protein complexes
Summary

– The effects appear to involve interaction of HSP-70 with protein complexes
– Prevents formation of large protein complexes
Summary

- The effects appear to involve interaction of HSP-70 with protein complexes
- Prevents formation of large protein complexes
- Suggests that activity lies in more sophisticated quaternary structure
Thank You

Spring Training starts in only 120 days.
Inhibition of δ–PKC
Inhibition of δ-PKC
Inhibition of δ-PKC
Inhibition of δ–PKC
Inhibition of δ–PKC
Inhibition of δ-PKC

[Diagram showing various pathways and molecules involved in the inhibition of δ-PKC, including p38 MAPK, Caspase-9, Bak, Bax, Cyt-C, PKCδ, c-abl, Bcl-2, Beclin-1, JNK, Cdk1, Cyclin D1, Akt, ERK, Autophagy, Apoptosis, Topoisomerase II, hnRNP-K, p53, c-abl, Rad9, Lamin, DNA-PK, and Mcl-1.]
Inhibition of δ-PKC
Inhibition of δ-PKC
δPKC In ARDS
PKC In ARDS

- Endothelial Activation
PKC In ARDS

- Endothelial Activation
- Neutrophil Adherence
PKC In ARDS

- Endothelial Activation
- Neutrophil Adherence
- Chemokine Production
PKC In ARDS

- Endothelial Activation
- Neutrophil Adherence
- Chemokine Production
- Neutrophil Diapedesis and Activation
PKC in ARDS

- Endothelial Activation
- Neutrophil Adherence
- Chemokine Production
- Neutrophil Diapedesis and Activation
- Necrosis and/or Apoptosis
Protection against Sepsis–induced Lung Injury by Selective Inhibition of Protein Kinase C

DAPI  MPO  Merge

2CLP + PBS

Sham

2CLP + TAT–δ–PKC
Protection against Sepsis–induced Lung Injury by Selective Inhibition of Protein Kinase C

2CLP + PBS

Sham

2CLP + TAT–δ–PKC
Protection against Sepsis–induced Lung Injury by Selective Inhibition of Protein Kinase C


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Thank You
These are abnormalities that can arise in many tissues in response to many insults. One would think that somehow evolution would provide protection. Is there something that might be damaged in ARDS that effects each of these?
Therapeutic Targets

Something like.....
Therapeutic Targets

Something like…..

Heat Shock Proteins
What Are Heat Shock Proteins?
What Are Heat Shock Proteins?
– Part of the phylogenetically conserved Heat Shock Response
What Are Heat Shock Proteins?
– Part of the phylogenetically conserved Heat Shock Response

Lowest Organism—Amoebas
What Are Heat Shock Proteins?
– Part of the phylogenetically conserved Heat Shock Response

Lowest Organism–Amoebas
Highest Organism–Humans
What Are Heat Shock Proteins?
– Part of the phylogenetically conserved Heat Shock Response

Not Tina Fey

Everything In Between
Impaired Type II Cell Proliferation

Impaired Type II Cell Proliferation

Impaired Type II Cell Proliferation

Impaired Type II Cell Proliferation

Impaired Type II Cell Proliferation

Impaired Type II Cell Proliferation

Impaired Type II Cell Proliferation

Impaired Type II Cell Proliferation


- AdHSP decreases E₂F DNA Binding
- AdHSP decreases Rb phosphorylation
Impaired Type II Cell Proliferation


- AdHSP decreases E$_2$F DNA Binding
- AdHSP decreases Rb phosphorylation
Impaired Type II Cell Proliferation


- AdHSP decreases E₂F DNA Binding
- AdHSP decreases Rb phosphorylation
Impaired Type II Cell Proliferation


Pro-SP-C Staining

Pro-SP-C (40x)

Pro-SP-C (100x)
Impaired Type II Cell Proliferation

Impaired Type II Cell Proliferation


Pro-SP-C
(40X)

Pro-SP-C
(100X)
Why HSP–70 Expression Might Be Important in ARDS

- **Heat shock** protects cultured Type II Cells from oxidant stress

- Animals demonstrate increased resistance to endotoxemia if heat treated before or parallel to the insult.

- Prior heat–treatment improves outcome from PL$_{A1}$ mediated ALI or systemically–induced ARDS in rats.
An Animal Model of ARD – 2CLP in Rats

YG Weiss et al, Anesthesiol., 2001
One would presume that expression of hsp–70 should increase in experimental ARDS.
One would presume that expression of hsp–70 should increase in experimental ARDS

BUT IT DOESNT
Pulmonary Hsp70 Expression after 2CLP

The Model – TAT-Hsp

<table>
<thead>
<tr>
<th></th>
<th>T₀</th>
<th>T₂₄</th>
<th>T₄₈</th>
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<tr>
<td>PBS</td>
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<td>TAT-HSP</td>
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AdHSP Attenuates NF-κB DNA Binding Activity and Intranuclear Abundance

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*
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AdHSP Attenuates NF-κB DNA Binding Activity and Intracellular Abundance


*
AdHSP Attenuates NF-κB DNA Binding Activity and Intranuclear Abundance

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**p<0.01 – 2CLP PBS/PBS vs HSP, *p<0.05-
HSP vs T₀**
AdHSP Attenuates NF-κB DNA Binding Activity and Intranuclear Abundance


**p<0.01 – 2CLP PBS/PBS vs HSP, *p<0.05 - HSP vs T₀**
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**p<0.01 – 2CLP PBS/PBS vs HSP, *p<0.05 - HSP vs T₀**
AdHSP Attenuates NF-κB DNA Binding Activity and Intranuclear Abundance


**p<0.01 – 2CLP PBS/PBS vs HSP, *p<0.05- HSP vs T₀**
Hsp70 Reduces But Does Not Abolish \( \mathrm{I\kappa \beta} \) Phosphorylation

YG Weiss, Z Bromberg, N Raj, Nichelle MS, J Raphael, P Goloubinoff, Y Ben-Neriah,
Hsp70 Reduces But Does Not Abolish IκBα Phosphorylation

YG Weiss, Z Bromberg, N Raj, Nichelle MS, J Raphael, P Goloubinoff, Y Ben-Neriah,
Hsp70 Reduces but does not Abolish IKKβ Phosphorylation

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YG Weiss, Z Bromberg, N Raj, Nichelle MS, J Raphael, P Goloubinoff, Y Ben-
Inhibition of Inflammation
NF-κB as a Surrogate

AdHSP Blocks… NF-κB DNA Binding

TNF
TNFR1

IKKγ
IKKα
IKKβ

p65
p50
IkBa

Ubiquitin Ligase

26S Proteosome

Pre-mRNA

AdHSP NF-κB Responsive Gene

p65
p50
IkBa

Ubiquitin

Ubiquitin Ligase

AdHSP Blocks… NF-κB DNA Binding
AdHSP Blocks NF-κB Nuclear Translocation

Pre-mRNA

AdHSP

NF-κB Responsive Gene

IKKγ
IKKα
IKKβ

Ubiquitin

TNF

TNFR1

Ubi

p65

p50

IkBα

IkBα

p50

Ubiquitin Ligase

26S Proteosome

AdHSP Blocks...
AdHSP Blocks... IkBa Phosphorylation
AdHSP Blocks... IKKα Phosphorylation
Loss of Type I Cells

Aquaporin-1 Staining

Loss of Type I Cells

Aquaporin-1 Staining

Loss of Type I Cells

Aquaporin-1 Staining

Aschkenasy G, Bromberg Z, Raj N, Deutschman CS, Weiss YG. Plos Med In press
Loss of Type I Cells
Apoptosis
G Aschkenasy, Z Bromberg, NR Raj, CS Deutschman, YG Weiss, Plos Med, In Press

- AdHSP impairs caspase - 8 and - 9
Loss of Type I Cells

Apoptosis

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- AdHSP impairs caspase-8 and -9 activity

Apaf-1-CARD dissociates from Pro-caspase-9 in the presence of Hsp70:
Detection of the cleaved caspase-9

5 min 10 min 20 min 30 min 5 min 10 min 20 min 30 min

2CLP PBS 2CLP AdHSP
Loss of Type I Cells
Apoptosis
G Aschkenasy, Z Bromberg, NR Raj, CS Deutschman, YG Weiss, Plos Med, In Press

– AdHSP impairs caspase-8 and -9 activity

Apaf-1-CARD dissociates from Pro-caspase-9 in the presence of Hsp70:
Detection of the cleaved caspase-9

![Image showing a gel with bands for different time points and conditions: 5 min, 10 min, 20 min, 30 min. The conditions are 2CLP PBS, 2CLP AdHSP.]
Apaf-1-CARD dissociates from Pro-caspase-9 in the presence of Hsp70: Detection of the cleaved caspase-9

- AdHSP impairs caspase-8 and -9 activity

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Apoptosis
G Aschkenasy, Z Bromberg, NR Raj, CS Deutschman, YG Weiss, Plos Med, In Press

- AdHSP impairs interaction between caspase 9 and Apaf
Loss of Type I Cells
Apoptosis
G Aschkenasy, Z Bromberg, NR Raj, CS Deutschman, YG Weiss, Plos Med, In Press

– AdHSP blocks translocation of activated Caspase – 3 into the nucleus

[Image of immunohistochemical staining and Western blot analysis showing the expression of Pro-Caspase-3 and Cleaved Caspase-3 in nuclear and cytosolic extracts under different conditions (2CLP PBS, 2CLP AdHSP, 2CLP AdGFP)].
Loss of Type I Cells
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– AdHSP blocks Caspase-8/Caspase-9 Interaction

![Image showing Western blots for Caspase-8 and Caspase-9]

- 2CLP PBS
- 2CLP AdHSP
- MLE-12 +TNF
- MLE-12 +TNF+AdHSP
- A549 +TNF
- A549 +TNF+AdHSP
- 2CLP PBS
- 2CLP AdHSP

**β-actin**

**IP Caspase-8**

**IP Caspase-9**

**B.**
Loss of Type I Cells
Apoptosis
G Aschkenasy, Z Bromberg, NR Raj, CS Deutschman, YG Weiss, Plos Med, In Press

- AdHSP stabilizes smaller molecular weight complexes containing Caspase – 3

Gel Filtration Chromatography
IP Caspase-9

<table>
<thead>
<tr>
<th>Molecular Weight</th>
<th>Apaf-1</th>
<th>Caspase-9</th>
<th>Caspase-8</th>
<th>Caspase-3</th>
<th>Hsp70</th>
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<tbody>
<tr>
<td>1500-900 kDa</td>
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<td>600-400 kDa</td>
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2CLPES, 2CLPAdHSP
Loss of Type I Cells
Apoptosis
G Aschkenasy, Z Bromberg, NR Raj, CS Deutschman, YG Weiss, Plos Med, In Press

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Gel Filtration Chromatography
IP Caspase-9

1500-900 kDa  600-400 kDa  400-300 kDa  200-100 kDa

Apaf-1
Caspase-9
Caspase-8
Caspase-3
Hsp70

2CLPPBS  2CLPPBS  2CLPPBS  2CLPPBS  2CLPPBS  2CLPPBS  2CLPPBS AdHSP
Loss of Type I Cells
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- AdHSP stabilizes smaller molecular weight complexes containing Caspase-3
AdHSP attenuates 2CLP-induced pulmonary apoptosis.
AdHSP attenuates 2CLP-induced pulmonary apoptosis.

AdHSP impairs caspase – 8 and – 9 activation and activity, impairs interaction between caspase – 9 and Apaf, blocks translocation of activated Caspase – 3 into the nucleus and stabilizes lower molecular weight complexes containing Caspases –8, –9.
The Pathological Basis of Acute Lung Injury

Lung infection
Aspiration
Sepsis
Multiple trauma, shock
Other insults

ACUTE LUNG INJURY

Lungs

Resolution of edema; repair of alveolar-capillary membrane

Persistence and progression of injury
- Multiple organ failure
- Pulmonary fibrosis
- Pulmonary vascular destruction

- Alveolar-capillary membrane injury
- Inflammation
- Increased permeability pulmonary edema

The Pathological Basis of Acute Lung Injury

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