Apoptosis and necrosis: detection, discrimination and clearance

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4 September 2009

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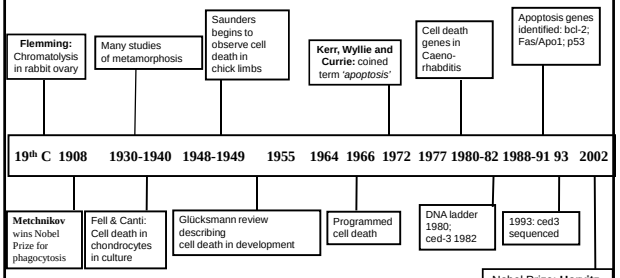
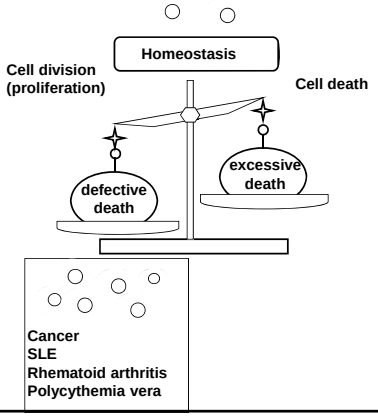
History of cell death field

description — *"PCD",morphology* — *genetics and biochemistry*



Cell death: history and future. Adv Exp Med Biol. 2008 Zakeri and Lockshin

Why cell death is important to study?



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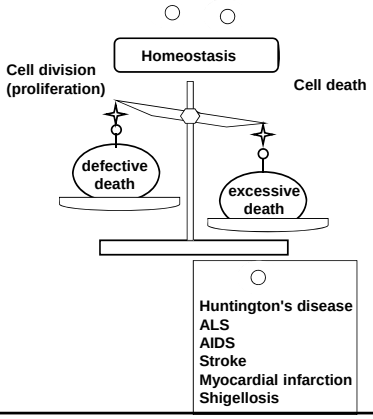

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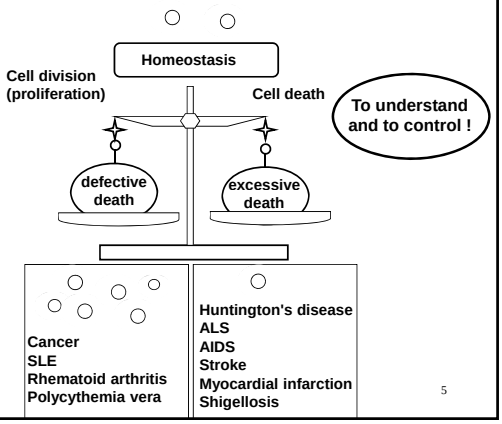
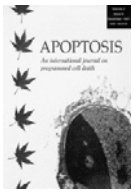


Many ways to die

- apoptosis: one particular morphological, biochemical, and functional (!) form of cell death
- other types of cell death are
  autophagic cell death
  - necrotic cell death
  - mitotic catastrophe
  - anoikis
  - excitotoxicity
  - wallerian degeneration
  - cornification



Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Kroemer et al. Cell Death and Differentiation 2009

Necrotic versus apoptotic cell death

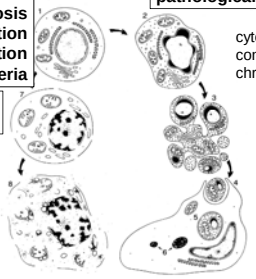
ischemia reperfusion
hypoxia
neuronal cell death
liver cirrhosis
organ transplantation
viral infection
Salmonella-infected bacteria

rounding of cell swelling of organelles

osmotic shock

plasma membrane permeablization (lysis)

release of intracellular content



developmental cell death
homeostasis and cell renewal
pathological conditions

cytoplasmic, nuclear condensation chromatin margination

membrane blebbing

cell implosion and disintegration in apoptotic bodies

rapid recognition and engulfment by phagocytic cells

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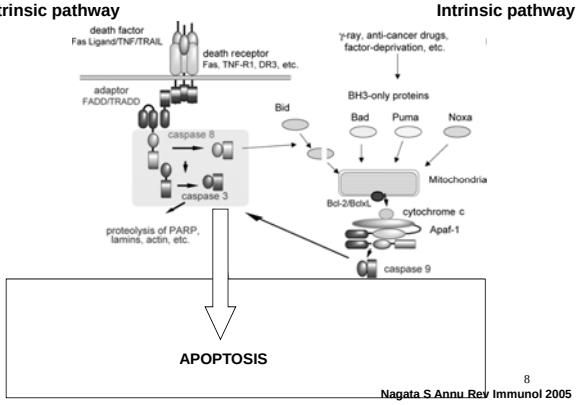
Kerr, Trends in Cell Biology, 1995

Apoptotic pathways

Extrinsic pathway **Intrinsic pathway**



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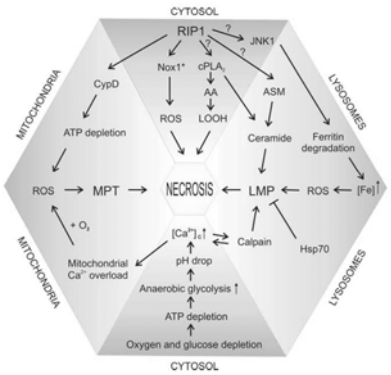
Nagata S Annu Rev Immunol 2005

Necrosis is also programmed



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Vanlangenakker et al Curr Mol Med 2008

How could we distinguish apoptosis from necrosis?

- Cell morphology
- Surface markers
- Intracellular markers
- Extracellular markers
- Phagocytosis

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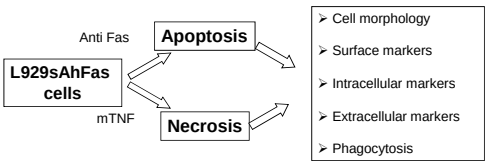
Model system



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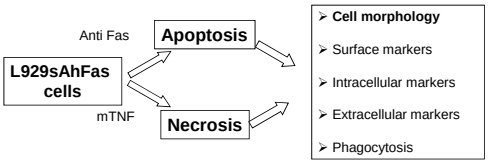
Model system



Cell morphology:

Time lapse microscopy
Transmission electron microscopy
Flow fluorocytometry

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Methods for analysis of cell morphology: Time-lapse microscopy

- DIC optics create a virtual relief image that allows morphological analysis of transparent objects
- Morphological changes that are specific for apoptosis or necrosis
- The duration and order of onset of subcellular events (e.g. rounding up of cells and formation of apoptotic bodies)

Optimization parameters to reduce phototoxicity and photobleaching:

- Lamp intensity
- Opening of filed diaphragm
- Number of *z* sections
- Time frame
- Camera settings (exposure time)

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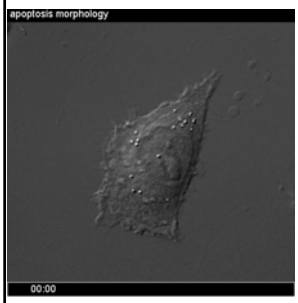
Methods for analysis of cell morphology: Time-lapse microscopy

Distinct morphological features of apoptotic versus necrotic cell death



- Rounding up of the cells
- Blebbing
- Formation of apoptotic bodies
- Formation of a balloon-like structure

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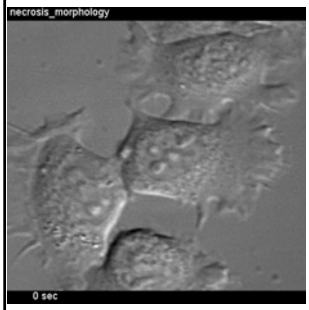
Methods for analysis of cell morphology: Time-lapse microscopy

Distinct morphological features of apoptotic versus necrotic cell death



- Cellular swelling
- Absence of blebbing and apoptotic bodies

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Methods for analysis of cell morphology: Time-lapse microscopy

Combination of DIC with epifluorescence mode allows:

- To use of fluorescent probes
- To link specific morphological features of cell death with particular molecular or subcellular cell death events

Fluorescence probes:

- Propidium iodide (PI), DAPI or Sytox family of dyes to determine plasma membrane permeability
- Annexin V-Alexa Fluor 488 to monitor phosphatidylserine exposure
- LysoTracker to visualize lysosomal integrity
- Tetramethylrosamine (TMRM) to measure mitochondrial depolarization
- Carboxy-H2DCFDA to detect production of reactive oxygen species

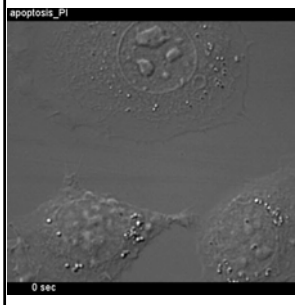
Methods for analysis of cell morphology: Time-lapse microscopy

Probes: DAPI, PI, 7AAD, Sytox family of dyes

- Exclusion dies are extruded by healthy cells, yet are taken up by cells with ruptured plasma membrane
- Routinely employed in several costaining protocols
- Possible to use in flow cytometry
- Unable per se to distinguish between apoptotic and necrotic cell death

Methods for analysis of cell morphology: Time-lapse microscopy

Propidium iodide (PI)



- Secondary necrotic cells have fragmented or condensed nuclei
- PI homogenously stains the nucleic acids content due to its binding to DNA by intercalating between the bases

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Methods for analysis of cell morphology: Time-lapse microscopy

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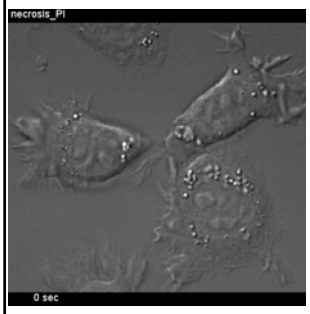

- Primary necrotic cells have uncondensed nuclei

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Methods for analysis of cell morphology: transmission electron microscopy

Advantages:

- **High resolving power (0.1-0.4 nm)**
- **Detection of subtle changes in organelle ultrastructure that occur early in the cascade of events leading to cell death**
- **Irreplaceable for an extremely precise (co)localization of proteins (immunoelectron microscopy)**

Disadvantages:

- **Inappropriate for large-scale quantitative applications**
- **Expensive, time consuming**

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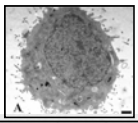
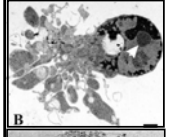
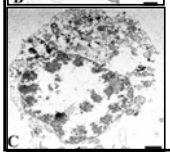
Methods for analysis of cell morphology: transmission electron microscopy



Leaving cell
- Microvilli protruding from the entire surface
- Smoothly outlined nucleus with chromatin in the form of heterochromatin
- Well-preserved cytoplasmic organelles



Apoptotic cell
- Sharply delineated masses of condensed chromatin
- Convolution of the cellular surface
- Formation of apoptotic bodies.
- Presence of the nucleolus (arrow head)



Necrotic cell
- Clumps of chromatin with ill-defined edges
- Swollen mitochondria
- Loss of plasma membrane integrity

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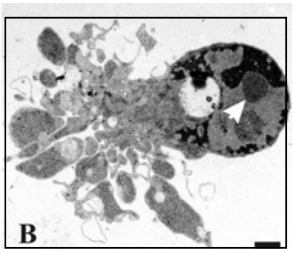
Krysko et al Methods 2008

Methods for analysis of cell morphology: transmission electron microscopy



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Krysko et al Methods 2008

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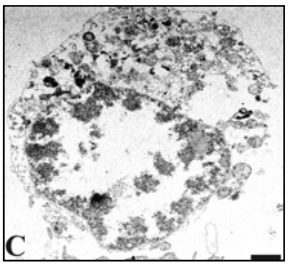

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Krysko et al Methods 2008

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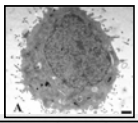

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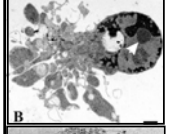

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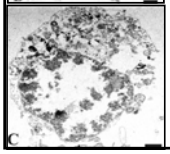


Necrotic cell
- Clumps of chromatin with ill-defined edges
- Swollen mitochondria
- Loss of plasma membrane integrity

Krysko et al Methods 2008

Methods for analysis of cell morphology: flow fluorocytometry

Advantages:

- **Rapid acquisition of 10 000-100 000 events per sample**
- **Cell by cell analysis**
- **Observer bias eliminated**
- **Up to 12 different fluorescent signals**
- **High-throughput screening (96-well plate cytofluorometers)**

Disadvantages:

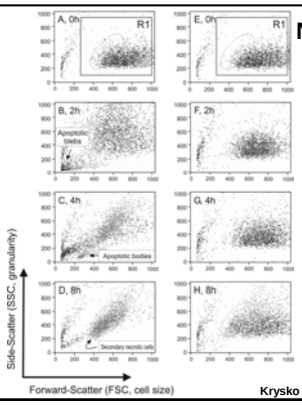
- **Unsuitable for the direct study of histological sections**

- **The forward scatter → cell size**
  Distinction between apoptotic blebs, apoptotic bodies and secondary necrotic cells
- **The side scatter → cell granularity**

Methods for analysis of cell morphology: flow fluorocytometry

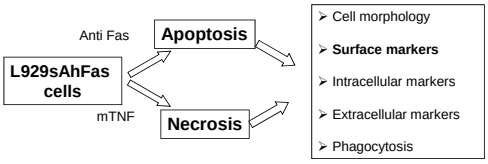
Apoptosis **Necrosis**

- Blebbing
- Apoptotic bodies
- Red: PI positive cells (secondary necrotic)
- Colocalization of secondary necrotic cells with primary necrotic (red)



Krysko et al Methods 2008

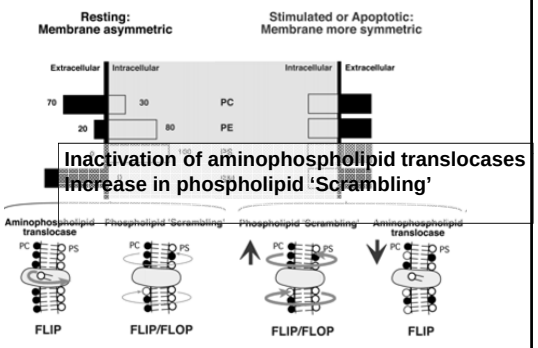
Model system



Surface markers:
Time-lapse microscopy
Flow fluorocytometry
Immunogold transmission electron microscopy
Immunocytochemistry

Example: phosphatidylserine exposure

Mechanisms for exposure of PS on the surface of activated or apoptotic cells



Inactivation of aminophospholipid translocases increase in phospholipid 'Scrambling'

PC, Phosphorylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin

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Gardai et al J Leukoc Biol. 2006

Methods for analysis of cell surface markers: phosphatidylserine exposure

- Annexin V binds PS in the presence of calcium
- Annexin V conjugated to FITC, PE, Cy3, Cy5, biotin, etc.
- High calcium concentration can affect the death process and kill cells (not more then 2.5mM)
- Unable to discriminate between apoptotic and necrotic cells
- Also exposed on autophagic cells

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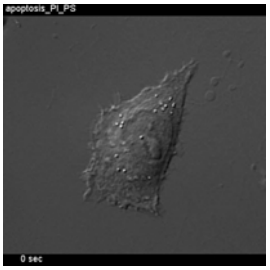
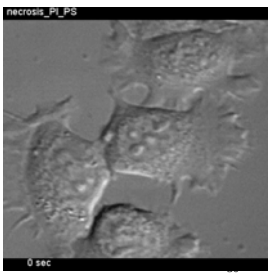
Methods for analysis of cell surface markers: phosphatidylserine exposure

Time-lapse microscopy:
Annexin V-Alexa488 for PS
PI for membrane integrity





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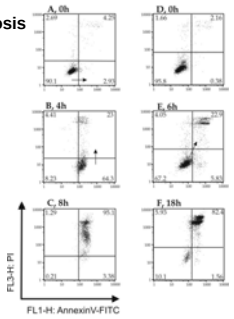
Methods for analysis of cell surface markers: phosphatidylserine exposure

Flow fluorocytometry
Annexin V-Alexa488 for PS
PI for membrane integrity

Apoptosis **Necrosis**

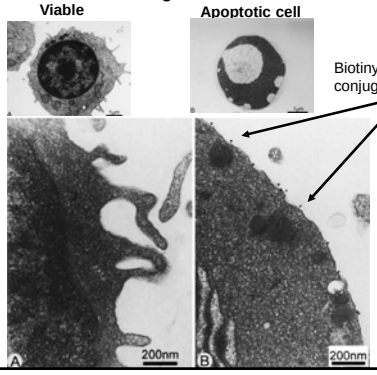


Krysko et al Methods 2008

Methods for analysis of cell surface markers: detection of phosphatidylserine exposure

Immunogold transmission electron microscopy

Viable **Apoptotic cell**



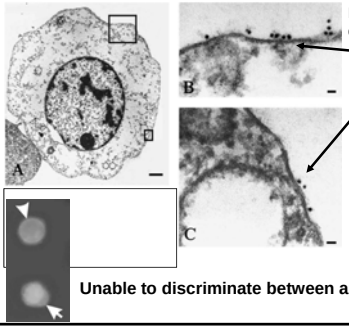
Biotinylated Annexin V+anti-biotin AB conjugated with 15 nm colloidal gold

Cornelissen et al Apoptosis 2002

Methods for analysis of cell surface markers: detection of phosphatidylserine exposure

Immunogold transmission electron microscopy

Necrotic cell



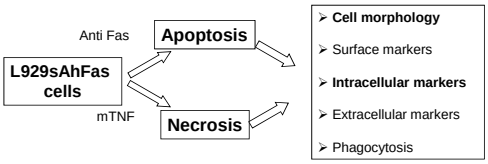
Biotinylated Annexin V+anti-biotin AB conjugated with 10 nm colloidal gold

JB6 cells treated with dsRNA

Unable to discriminate between apoptotic and necrotic cells

Krysko et al Apoptosis 2004

Model system

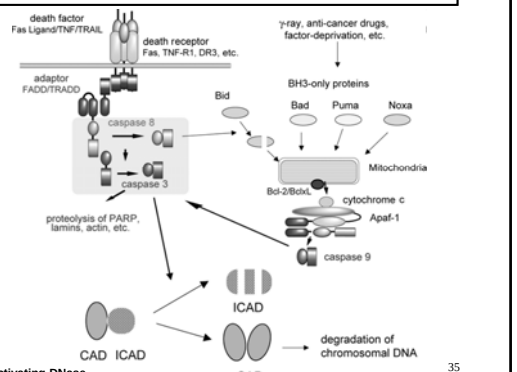


Intracellular markers:

Flow fluorocytometry
Western blotting
Fluorometry

Example: DNA fragmentation, caspase activity and PARP cleavage

Apoptotic cell signaling pathways leading to DNA degradation



CAD: caspase-activating DNase
ICAD: inhibitor of CAD

Nagata S Annu Rev Immunol 2005

Methods for analysis of intracellular markers: DNA fragmentation

- DNA fragmentation is a classical feature of apoptosis which is not present in necrosis
- DNA fragmentation and formation of high molecular weight (>50 kbp) and nucleosome-sized (200 bp) DNA fragments

Methods:

- Flow fluorocytometry
- Agarose gel electrophoresis for detection of DNA ladders
- The terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) technique for in vitro and in vivo use

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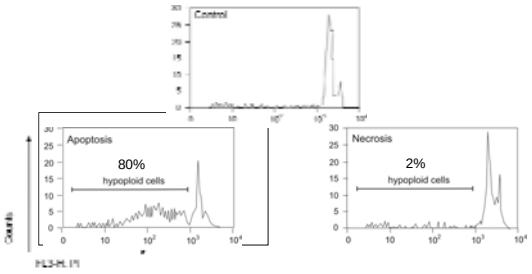
Methods for analysis of intracellular markers: DNA fragmentation

Flow fluorocytometry

- Based on detection of DNA hypoploidy after adding PI to the dying cells and permeabilizing them by freeze-thawing
- Quantitative
- Allows to discriminate between apoptosis and necrosis

Methods for analysis of intracellular markers: DNA fragmentation

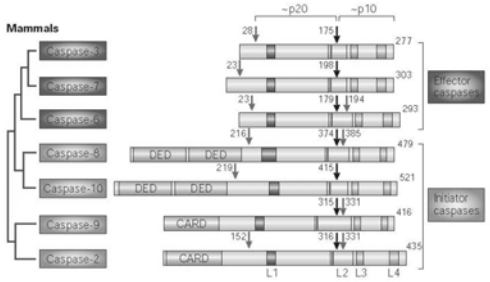
Flow fluorocytometry



Hypoploid DNA is absent in necrosis

Krysko et al Methods 2008

Methods for analysis of intracellular markers: caspase activation



Riedl and Shi Nat Rev Mol Cell Biol 2004

Methods for analysis of intracellular markers: caspase activation

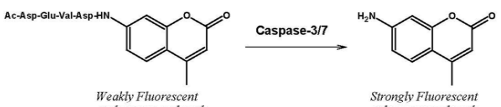
Fluorometry

Typical substrates contain amino acid composition corresponding to the cleavage site of a typical substrat coupled to 7-amino-4-methylcoumarin (AMC) or 7-amino-4-trifluoromethylcoumarin (AFC).

Ac-DEVD-AMC -	caspase-3/7
Ac-LEHD-AMC -	caspase-5
Ac-YVAD-AMC -	caspase-1/4
Ac-IETD-AMC -	caspase-8/6
Ac-WEHD-AMC -	caspase-1/4/5

Methods for analysis of intracellular markers: caspase 3/7 activation by fluorometry

Fluorometry: Proteolytic cleavage of Ac-DEVD-AMC



Weakly Fluorescent at shorter wavelength

Strongly Fluorescent at longer wavelength

ANASPEC

SensoLyte® Homogeneous AMC Caspase-3/7 Assay Kit

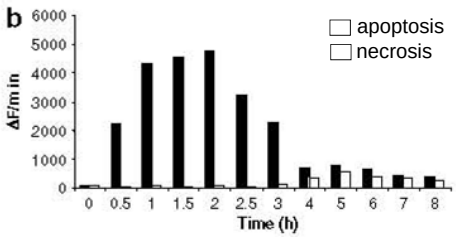
Can be detected at excitation/emission =354 nm/442 nm

Advantages:

- Discriminate between apoptosis and necrosis
- High-through put screening (96-well or 384-well plate)
- Enzymatic measurements of caspase activities should be combined with western blotting to identify the presence and activation status of the caspases
- Does not discriminate between activation of caspase-3 and 7

Methods for analysis of intracellular markers: caspase 3/7 in apoptosis vs. necrosis

Fluorometry using Ac-DEVD-AMC as a probe

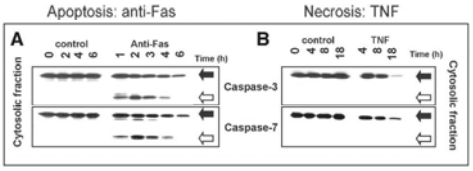


Activation of caspase-3/7 in apoptosis and not in necrosis

Krysko DV et al Cell Death Different 2006

Methods for analysis of intracellular markers: caspase 3/7 processing in apoptosis vs. necrosis

Western blotting



⇦ processed p20 subunit
⇦ precursor procaspase

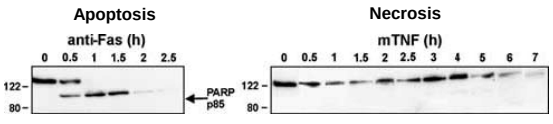
Antibodies are also available that specifically recognize the activated form of caspases (PharMingen) which could be used for FACS and ICH

Denecker G et al Cell Death Different 2001

Methods for analysis of intracellular markers: PARP-1 cleavage in apoptosis vs. necrosis

Western blotting

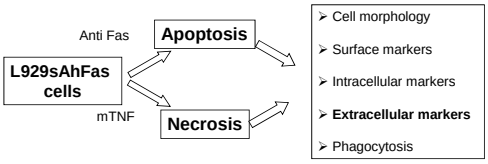
- Poly(ADP-ribose) polymerase-1 (PARP-1) is an enzyme implicated in DNA damage and repair mechanisms
- During apoptosis, PARP-1 is cleaved by caspase-3



Cleavage of PARP-1 from the native 116 kDa to 85 kDa is a hallmark of apoptosis which is absent in necrosis.

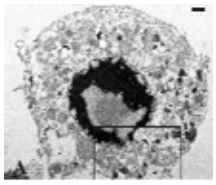
Krysko DV et al Cell Death Different 2006

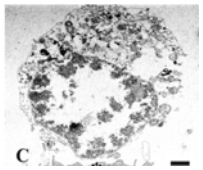
Model system



Extracellular markers:
Lactate dehydrogenase assay and caspase 3/7 by fluorometry
Cytokeratin-18 detection by ELISA

Secondary necrotic and primary necrotic cells: transmission electron microscopy





Secondary necrotic cell:

- Margination of chromatin
- Vacuolization of the cytoplasm
- Clearly damaged organelles
- Loss of plasma membrane integrity

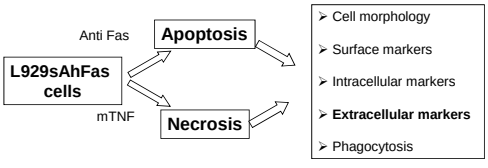
Primary necrotic cell:

- Ill-defined edges of the clumps of compacted chromatin
- Swollen mitochondria with matrix densities
- Dissolution of membranes
- Loss of plasma membrane integrity.

Release of different factors

Krysko DV et al Methods 2008
Krysko DV et al J Morphol 2003

Model system



Extracellular markers:
Lactate dehydrogenase assay and caspase 3/7 by fluorometry
Cytokeratin 18 detection by ELISA

Methods for analysis of extracellular markers: LDH release

Advantages:

- Allows large scale analysis
- Eliminates labeling of cells before experiments
- Eliminates safety issues of radioactivity
- Allows use of standard plate reader

Disadvantages:

- Does not discriminate between apoptotic and necrotic cell death
- The release of LDH activity can be related to the total No. of dead & lysed cells

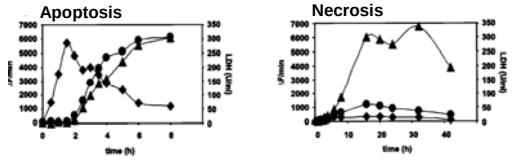
$$NAD^+ + \text{lactate} \rightarrow \text{pyruvate} + NADH$$

$$NADH + \text{INT} \rightarrow NAD^+ + \text{formazan (red)}$$

INT- tetrazolium salt
CytoTox 96 non-radioactive cytotoxicity assay from Promega

Methods for analysis of extracellular markers: LDH and caspase release

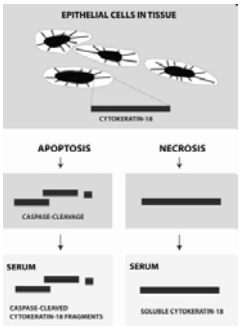
Ac-DEVD-amc cleavage activity in cytosol (◇), and supernatant (○)
LDH in supernatant (△)



- LDH does not discriminate between secondary necrosis and primary necrosis
- Caspases 3/7 are enzymatically active on Ac-DEVD-amc fluorogenic substrate in apoptosis and not in necrosis

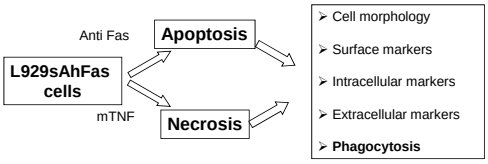
Denecker G et al Cell Death Different 2001

Methods for analysis of extracellular markers: cytokeratine-18



PEVIVA http://www.peviva.se/

Model system

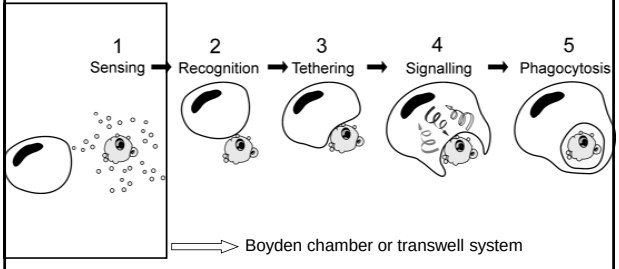


Phagocytosis of dead cells:
Confocal fluorescence and light microscopy
Transmission and scanning electron microscopy
Flow fluorocytometry

Macrophage and dendritic cell populations for study

- Monocyte-derived macrophages and dendritic cells (human)
  - Monocyte isolated from blood and cultured *in vitro* to acquire macrophage or dendritic cell characteristics
- Alveolar Macrophages (human/mice)
  - From bronchoalveolar lavage
- Peritoneal Macrophages (mice)
  - Either resident or elicited with inflammatory agent
- Bone-marrow-derived macrophages and dendritic cells (mice)
  - Mf expanded from progenitors over 7-10 days by M-CSF
  - DCs Expanded from progenitors over 7-10 days by IL-4 and GM-CSF
- Macrophage cell lines (human/mice)

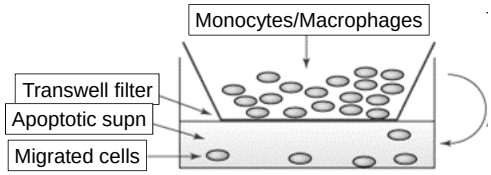
Phases in the interactions between dying cells and phagocytes



Gregory and Devitt Immunology 2004

Gregory and Pound in "Phagocytosis of dying cells", Springer publisher

Transwell assay to investigate chemotactic migration



- Cells above the membrane, chemoattractant is below → concentration gradient
- Pores 3-8 microns (large enough for the cells to squeeze through, small enough so they do not fall)
- Give time to migrate, fix and stain the membrane, and count cells at the bottom
- Disadvantage: cannot trace migration
- Advantage: good to view population migration

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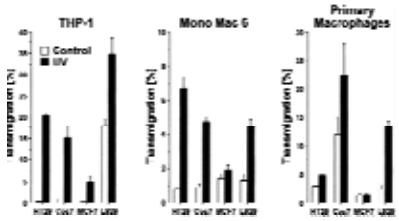
Grimsley and Ravichandran Trends Cell Biol 2003

Attraction of monocytic cells to supernatants of apoptotic cells



Supernatants of apoptotic cells are able to induce migration

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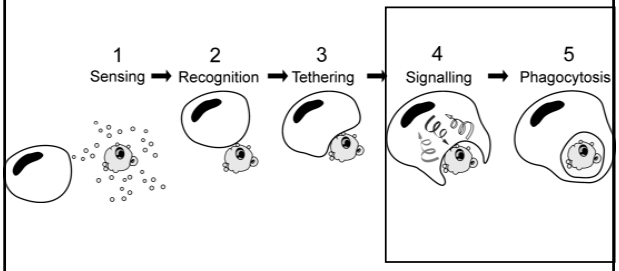
Lauber et al Cell 2003

Phases in the interactions between dying cells and phagocytes



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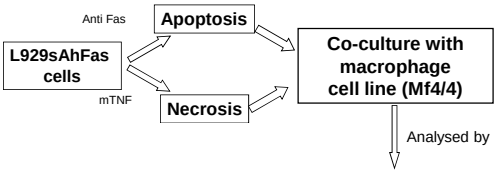
Gregory and Devitt Immunology 2004

Gregory and Pound in "Phagocytosis of dying cells", Springer publisher

How can we measure phagocytosis?

- Light microscopy
- Fluorescence (confocal) microscopy
- Transmission and Scanning Electron Microscopy
- Time-lapse microscopy
- Flow fluorocytometry

Internalization mechanisms of apoptotic and necrotic cells



- Light microscopy
- Fluorescence (confocal) microscopy
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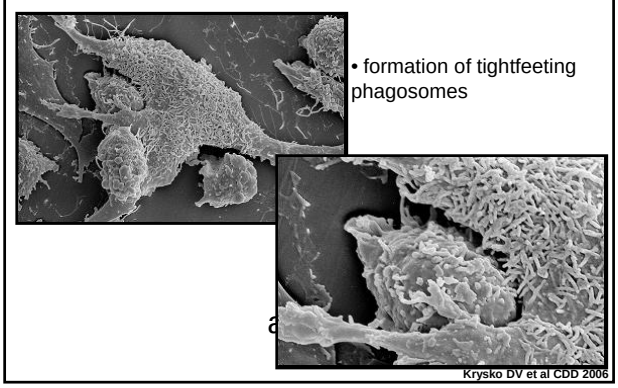
- Flow fluorocytometry

Internalization mechanisms of apoptotic cells



• formation of tightfeeting phagosomes

Krysko DV et al CDD 2006

How can we measure phagocytosis?

Conclusions:

Microscopy:
-Discriminate internalization mechanisms

-Tedious
-Time consuming
-Observer bias
-Difficult to be certain of particle internalisation

Flow fluorocytometry:
-Rapid
-Cell by cell analysis
-Observer bias eliminated
-Still can be difficult to distinguish internalisation from binding

Dual color flow cytometry

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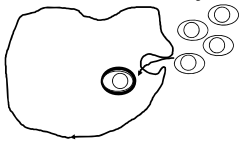
In vitro phagocytosis assay for dual color flow fluorocytometry

How the assay works:

- Target cells and macrophages are labeled with a fluorescent probes Cell Tracker Green and Orange, respectively
- The cell death is induced in the target cells (e.g. aFas antibodies)
- The dead target cells are mixed with phagocytes (at certain ratio) so phagocytosis takes place
- After certain time (e.g. 2 hours) non-ingested cells are washed away and macrophages are detached and analyzed by flow cytometry
- Double stained macrophages means that dead cells are taken up

red: macrophages



green: apoptotic cells

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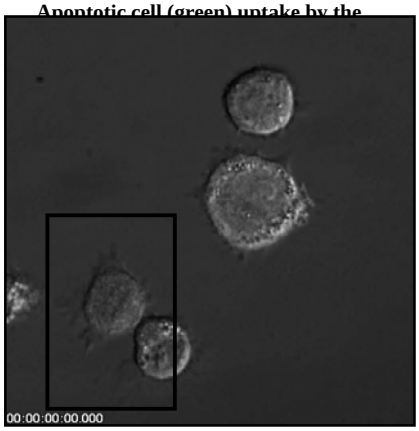
Apoptotic cell (green) uptake by the



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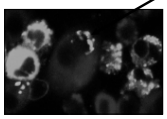
Dual color flow fluorocytometry measure clearance of dying cells

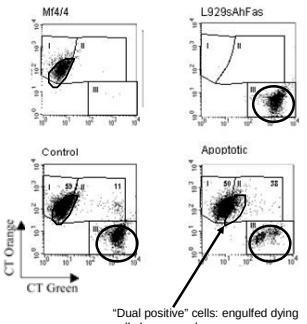
Flow cytometry:
-Rapid
-Cell by cell analysis
-Observer bias eliminated
-Still can be difficult to distinguish internalisation from binding

Confocal images of "dual positive" cells



green:
apoptotic cells

red:
macrophages



"Dual positive" cells: engulfed dying cells by macrophages

To distinguish apoptosis from necrosis:

- Morphological features of cell death could be different depending on the tissue/cell type
- PI/PS is not discriminative between apoptosis and necrosis
- To use combination of techniques to discriminate different types of cell death

Vandenabeele's group
« In cell death we trust »

Further reading

- **Apoptosis and necrosis: detection, discrimination and phagocytosis.** Krysko DV, Vanden Berghe T D'Herde K, Vandenabeele P. Methods. 2008 Mar;44(3):205-21.
- **Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes.** Galluzzi L et al. Cell Death Different 2009
- **Phagocytosis of dying cells from molecular mechanisms to human diseases.** Springer Publisher, 2009, Editors: Krysko DV and Vandenabeele P

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