Cell death: apoptosis, necrosis, autophagy
Apoptosis is an altruistic form of cell suicide.

In a multicellular organism, individual cells commit suicide when intrinsic or extrinsic conditions lead them to transformations they “sense” as potentially harmful for the whole organism. (e.g.: DNA damage, oncogene activation, receptor stimulation….)

The term apoptosis has been introduced in the literature in 1972 by Kerr, Currie e Wyllie to describe a series of morphological modifications associated with the death in some cells and tissues.
# Categorization of cell death

<table>
<thead>
<tr>
<th>Cell Death Mode</th>
<th>Morphological Features</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis</td>
<td>Rounding-up of the cell&lt;br&gt;Retraction of pseudopodes&lt;br&gt;Reduction of cellular and nuclear volume (pyknosis)&lt;br&gt;Nuclear fragmentation (karyorrhexis)&lt;br&gt;Minor modification of cytoplasmic organelles&lt;br&gt;Plasma membrane blebbing&lt;br&gt;Engulfment by resident phagocytes, in vivo</td>
<td>‘Apoptosis’ is the original term introduced by Kerr et al. to define a type of cell death with specific morphological features. Apoptosis is NOT a synonym of programmed</td>
</tr>
<tr>
<td>Autophagy</td>
<td>Lack of chromatin condensation&lt;br&gt;Massive vacuolization of the cytoplasm&lt;br&gt;Accumulation of (double-membraned) autophagic vacuoles&lt;br&gt;Little or no uptake by phagocytic cells, in vivo</td>
<td>‘Autophagic cell death’ defines cell death occurring with autophagy, though it may misleadingly suggest a form of death occurring by autophagy as this process often promotes cell survival</td>
</tr>
<tr>
<td>Necrosis</td>
<td>Cytoplasmic swelling (oncasis)&lt;br&gt;Rupture of plasma membrane&lt;br&gt;Swelling of cytoplasmic organelles&lt;br&gt;Moderate chromatin condensation</td>
<td>Necrosis’ identifies, in a negative fashion, cell death lacking the features of apoptosis or autophagy. Note that necrosis can occur in a regulated fashion, involving a precise sequence of signals.</td>
</tr>
</tbody>
</table>
Apoptosis

- stimuli include signaling triggered by:
  - cell surface receptors, growth factor withdrawal, hypoxia, heat shock, DNA damage, viral infection, chemotherapeutic agents
- involved in many physiologic events:
  - embryogenesis, differentiation, homeostasis, aging, removal of defect and/or harmful cells
- cause for a variety of pathologic disorders:
  - neurodegenerative disease, immunodeficiency, autoimmune disease, and cancer
The Nobel Prize in Physiology and Medicine 2002

"for their discoveries concerning 'genetic regulation of organ development and programmed cell death'"

Sydney Brenner
1/3 of the prize
United Kingdom

H. Robert Horvitz
1/3 of the prize
USA

John E. Sulston
1/3 of the prize
United Kingdom

The Molecular Sciences Institute
Berkeley, CA, USA
b. 1927
(in Union of South Africa)

Massachusetts Institute of Technology (MIT)
Cambridge, MA, USA
b. 1947

The Wellcome Trust Sanger Institute
Cambridge, United Kingdom
b. 1942

http://nobelprize.org/medicine/laureates/2002/
Morphological modifications of a cell undergoing apoptosis or necrosis
Human Fibroblast in culture in early and advanced spontaneous apoptosis

Apoptosis in HL60 caused by camptothecin
APOPTOSIS
## Biochemical aspects: Methods of detection

<table>
<thead>
<tr>
<th>Cell death mode</th>
<th>Biochemical features</th>
<th>Methods of detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis</td>
<td>Activation of Bcl2 family proteins</td>
<td>IF microscopy - localization studies; WB with conformation specific Ab</td>
</tr>
<tr>
<td></td>
<td>Activation of caspases</td>
<td>Colorimetric-fluorogenic substrate-based assays in live cells or with lysates; WB with Ab for activation caspase-state or cleaved caspase; FACS/IF with fluorogenic substrates; FACS/IF cleaved caspases</td>
</tr>
<tr>
<td></td>
<td>mitochondrial transmembrane permeabilization</td>
<td>FACS/IF microscopy quantification with specific probes</td>
</tr>
<tr>
<td></td>
<td>Oligonucleosomal DNA fragmentation</td>
<td>DNA ladders FACS quantification of hypodiploid cells (sub-G1 peak) TUNEL assays</td>
</tr>
<tr>
<td></td>
<td>PS (Phosphatidylserine) exposure</td>
<td>FACS quantification of Annexin V binding</td>
</tr>
<tr>
<td></td>
<td>mitochondrial membrane permeabilization</td>
<td>IF (video) microscopy with Cyt c-GFP fusion protein Immunoblotting detection of IMS proteins (e.g., Cyt c) upon cellular fractionation</td>
</tr>
</tbody>
</table>
A schematic representation of the principle of detection of apoptosis by staining of plasma membranes with annexin.
Apoptosis - DNA Analysis

During apoptosis, calcium- and magnesium-dependent nucleases are activated which degrade DNA. This means that within the DNA there are nicks and fragmentation. We can detect these in three ways - using DNA analysis to look at a SubG1 peak, using strand break labelling (TUNEL) to detect broken DNA or using Hoechst binding to detect DNA conformational changes.

The Sub-G1 method relies on the fact that after DNA fragmentation, there are small fragments of DNA that are able to be eluted following washing in either PBS or a specific phosphate-citrate buffer. This means that after staining with a quantitative DNA-binding dye, cells that have lost DNA will take up less stain and will appear to the left of the G1 peak.
DNA fragmentation in apoptosis

- Chromatin
- DNA fragmentation
- Nucleosome
- Core histone
- DNA
- Endonucleolytic
- 180bp
- Agarose gel electrophoresis
DNA cleavage the ICAD/CAD complex

CAD: Caspase-activated Deoxyribonuclease
The TUNEL method is often used to detect strand breaks within DNA. TUNEL is an acronym for Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labelling. The enzyme Tdt is used to add dUTPs to the broken ends of the DNA, these can then be detected by antibodies with fluorochrome labels. The DNA may simultaneously be stained with propidium iodide so it is possible to tell from which phase of the cell cycle the cells are exhibiting strand breaks.
Cell-extrinsic and cell-intrinsic apoptotic signaling pathways

Nature Reviews | Cancer
Receptor-mediated apoptosis
Intrinsic apoptosis: the Bcl-2 family

Pro-apoptotic proteins can be subdivided as effectors (BAK, BAX, BOK) or BH3 only (signaling to the effectors)
Bcl-2 Is Expressed in Most Types of Cancer

Mitochondrial outer membrane permeabilization (MOMP) is a critical event in cell death. The process begins with BH3-only proteins interacting with BAX or BAK, leading to an exposure of the BH3 domain. This exposure promotes dimer formation, which can result in higher-order oligomerization. The formation of oligomers can lead to the creation of proteinaceous channels and lipidic pores in the mitochondrial outer membrane, allowing the release of cytochrome c from the intermembrane space (IMS).
Regulation of mitochondrial apoptosis

Healthy cell

BCL-XL

BAX

mitochondria

BH3 activator

penetration of outer membrane

BAX/BAK pore

conformational change of BAX

cytochrome c

BH3 neutralizer

Induction of apoptosis
...it is a matter of balance
First stage of apoptosome formation

- Apaf-1
- Cytochrome C

Recruitment of procaspase-9

Procaspe-9

Caspase Activation
Oncogenes can induce apoptosis
MYC is a very powerful oncogene

In this experiment, MYC is expressed in the beta islands in the pancreas under the control of insulin promoter: when MYC is on there is apoptosis of the beta cells. You need to block apoptosis to have cancer. It is impressive that in this case, there is a full transformation with the presence of new vessels.
Phosphorylation of p53Ser46 is associated with induction of apoptosis

By Oda et al., 2000 (modified)

* D’Orazi et al., Nat Cell Biol 2002
* Hofmann et al., Nat Cell Biol 2002
PUMA (p53 upregulated modulator of apoptosis) is a BH3-only protein. It directly binds and antagonizes all known antiapoptotic Bcl-2 family members to induce mitochondrial dysfunction and caspase activation.

The inhibitors of apoptosis proteins (IAPs) are inhibitors for apoptosis. IAPs regulate caspases through two conserved regions, the baculovirus IAP repeats (BIRs) and (RING) domains. BIRs bind to caspases, the RING domain can act as a ubiquitin-E3 ligase, leading to ubiquitylation of IAPs themselves and their pro-apoptotic IAP counterparts such as caspases.
p53 can act directly binding the anti-apoptotic proteins in the mitochondrial membrane.
DNA damage

P53 stabilization

Nuclear accumulation
- Regulation of gene expression
  - PUMA expression

Cytoplasmic accumulation
- Bcl-xL sequesters p53
  - PUMA liberates p53
  - P53 activates BAX

APOPTOSIS
TNF-related apoptosis-inducing ligand (TRAIL, also known as Apo2L) is a cytokine secreted by most tissue cells. Their receptors are preferentially expressed in cancer cells. Normal cells are less responsive to TRAIL due to the abundancy of DcR1 and DcR2 in comparison to cancer cells.

...possesses the unique capacity to induce apoptosis selectively in cancer cells in vitro and in vivo. This exciting discovery provided the basis for the development of TRAIL-receptor agonists (TRAs), which have demonstrated robust anticancer activity in a number of preclinical studies.
Upon binding of trimerized TRAIL to TRAIL-R1/2, the adaptor molecule FADD is recruited via homotypic DD interaction. Subsequently, FADD recruits pro-caspase-8/10 molecules via their respective DEDs. The E3 ligase Cullin3 stabilizes DISC formation by polyubiquitination of caspase-8. Different forms of cFLIP can inhibit DISC formation by competing with caspase-8/10 for binding to FADD. TRAF2 has been suggested to negatively regulate DISC activity by promoting K48-linked ubiquitination and subsequent proteasomal degradation of caspase-8.
Agonists of TRAIL Receptors are already in clinical trials
Two Different Types of Cell Death

**Necrosis**
- Normal
- Reversible swelling
- Inversible swelling
- Disintegration

**Mitochondrial changes**
- Chromatin pattern conserved

**Apoptosis**
- Normal
- Condensation (cell blebbing)
- Fragmentation
- Secondary necrosis

**Intact membranes**
- Apoptotic bodies
<table>
<thead>
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</tr>
</thead>
<tbody>
<tr>
<td><strong>Necrosis</strong></td>
<td>Activation of calpains</td>
<td>Colorimetric/fluorogenic substrate-based assays of cell lysates in</td>
</tr>
<tr>
<td></td>
<td>Activation of cathepsins</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Drop of ATP levels</td>
<td>Luminometric assessments of ATP/ADP ratio</td>
</tr>
<tr>
<td></td>
<td>RIP1 phosphorylation</td>
<td>Immunoblotting with -specific antibodies</td>
</tr>
<tr>
<td></td>
<td>RIP1 ubiquitination</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LMP, lysosomal membrane permeabilization</td>
<td>FACS quantification with lysomorphotropic probes</td>
</tr>
<tr>
<td></td>
<td>Plasma membrane rupture</td>
<td>Colorimetric/fluorogenic substrate-based assays of culture supernatants in microtiter</td>
</tr>
<tr>
<td></td>
<td></td>
<td>plates to determine the release of cytosolic enzymatic activities (e.g., LDH)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FACS quantification with vital dyes</td>
</tr>
<tr>
<td></td>
<td>ROS overgeneration</td>
<td></td>
</tr>
<tr>
<td><strong>Autophagy</strong></td>
<td>Dependency on atg gene products</td>
<td>Genetic studies (e.g., knockout models, RNA interference, plasmid-driven overexpression systems)</td>
</tr>
<tr>
<td></td>
<td>LC3-I to LC3-II conversion</td>
<td>IF microscopy with GFP-LC3 fusion protein</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Immunoblotting with LC3-specific antibodies</td>
</tr>
</tbody>
</table>
Necrosis

Activated by calcium

CLP1: calcium-dependent cysteine-type endopeptidase  ASP: aspartic protease-like protein
Necrotic cells release immunogenic endogenous molecules that fall under the umbrella term “damage-associated molecular patterns” (DAMPs) (Garg et al., 2010; Krysko et al., 2011). They include, in the case of accidental necrosis, HMGB1, IL-1α, uric acid, DNA fragments, mitochondrial content, and ATP.
HMGB1 is a nuclear factor that binds DNA in a not-sequence specific manner. It binds to the minor groove and helps the 90’ DNA bending thus favouring the interaction with the heteromeric complexes serving a pletora of functions (transcription, replication, VDJ recombination etc). It binds the nucleosomes, but, differently from the H1 that blocks them, HMGB1 helps their sliding and mobility. It was really a surprise to discover that HMGB1 is secreted from monocytes and released from the necrotic cells thus working as a pro-inflammatory cytokine. Necrotic cells deriving HMGB1 -/- mice induce a weaker inflammation than wt mice. In the case of apoptotic cells, the chromatin collapses in a condensed state that does not allow HMGB1 detachment and therefore there is not inflammation.
Structure and function of HMGB1.

A

p53 transactivation-binding domain (7–74)

TLR4-binding domain (89–108)

RAGE-binding domain (150–183)

N

A box

B box

Acidic tail

C

NLS1 (28–44)

NLS2 (179–185)

B

In the nucleus

- DNA chaperone
- Sustains nucleosome dynamics and chromosomal stability
- Modulates gene transcription and V(D)J recombination
- Participates in DNA repair and telomere maintenance

In the cytosol or mitochondria

- Binds Beclin 1 by C23 and C45
- Increases autophagy and inhibits apoptosis
- Regulates mitochondrial morphology and function

At the cell surface

- Promotes axonal sprouting and neurite outgrowth

At the extracellular fluid

- DAMP signaling
- Interacts with multiple receptors
- Forms heterocomplexes with other immune coactivators
- Regulates inflammation, immunity, migration, proliferation, metabolism, autophagy, and apoptosis

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Review

Cancer therapy in the necroptosis era

Z Su\textsuperscript{1,2,5}, Z Yang\textsuperscript{1,3,5}, L Xie\textsuperscript{3,5}, JP DeWitt\textsuperscript{2,5} and Y Chen\textsuperscript{4}
Necroptosis

✓ Evidence now reveals that necrosis can also occur in a regulated manner.

✓ The initiation of programmed necrosis, ‘necroptosis’, by death receptors (such as tumour necrosis factor receptor 1) requires the kinase activity of receptor-interacting protein 1 (RIP1; also known as RIPK1) and RIP3 (also known as RIPK3).

✓ Its execution involves the active disintegration of mitochondrial, lysosomal and plasma membranes.

✓ Necroptosis participates in the pathogenesis of diseases, including ischaemic injury, neurodegeneration and viral infection, thereby representing an attractive target for the avoidance of unwarranted cell death.
Facts
A plethora of cancer cell lines can undergo necroptosis by classic necroptosis inducers and existing chemotherapeutic agents.

Triggering necroptosis could be an alternative way to eradicate apoptosis-resistant cancer cells.

Intrinsic or acquired defects of necroptotic machinery are observed in many cancer cells.
Necroptosis players

The ubiquitin-editing system and initiator caspases such as caspase 8 modulate the molecular switches that dictate the biological response to TNFR1 activation.
TNF signaling depends on the context
TNFR1-elicited signalling pathways

Complex I - NF-κB activation

cIAPs — E3 ubiquitin ligases that were previously known as apoptosis inhibitors owing to their ability to interfere with caspase activation are recruited to complex I by TRAF2, which stabilizes them by preventing their polyubiquitylation.

cIAPs catalyse the addition of Lys63-linked polyubiquitin moieties to Lys377 of RIP1. Lys63-polyubiquitylated RIP1 provides a docking site for transforming growth factor-β-activated kinase 1 (TAK1), TAK1-binding protein 2 (TAB2) and TAB3, which together (the TAK1-TAB2-TAB3 complex) constitute the apical stimulator of the canonical nuclear factor-κB (NF-κB) activation pathway.

On deubiquitylation of RIP1 by the Lys63-deubiquitylating enzyme cylindromatosis RIP1 along with RIP3 is recruited in complex II.

Vandenabeele et al. 2010
The interplay between caspase 8 and RIP1 activity determine the outcome of many death stimuli.

P Kreuzaler and CJ Watson, 2012 Nature Reviews/Cancer
Molecular pathways of necroptosis

MLKL (mixed lineage kinase domain like *pseudokinase*)
Execution of necroptosis

ATP-consuming processes;
ROS production by mitochondrial respiratory complex I
Involvement of LMP in the execution of necroptosis.

Vandenabeele et al. 2010
Necroptosis summary

• The kinase activities of RIPK1 and RIPK3 are crucial for Necroptosis

• The FADD/caspase-8 apoptotic platform negatively regulates RIPK1/3-mediated necroptosis

• RIPK1 and RIPK3 kinase activities contribute to pathogenesis in IR injury, pancreatitis, photoreceptor cell loss and intestinal epithelial cell loss

• RIPK1 and RIPK3 kinase activities contribute to an appropriate immune response during viral and microbial Infections

• Some forms of regulated necrosis act independently of RIPK1 or RIPK3 kinase activity.
Pro-necroptotic cancer therapy

- Triggering necroptosis is an alternative way to eradicate apoptosis-resistant cancer cells

- Cancer cells may evade necroptosis
  - Down-regulation of RIP1, RIP3, and MLKL
  - Mutations of RIP1, RIP3, and MLKL
  - Hypoxia-induced glycolytic metabolism

- Possible off-target effect
  - Normal cells should have the intact necroptotic machinery
  - Necroptosis is involved in the pathogenesis of some inflammatory diseases

How to achieve a successful necroptosis-based therapy?

- Conduct a genetic detection of RIP1, RIP3, and MLKL before using a pro-necroptotic drug.
- Combine with other therapeutic strategies (e.g., antagonists of hypoxia, inhibitors of anaerobic glycolysis, and heat therapy)
- Develop pro-necroptotic drugs that directly target MLKL