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Programmed Cell Death: Molecular Mechanisms and Implications for Safety Assessment of Nanomaterials

FERNANDO TORRES ANDÓN AND BENGT FADEEL*
Division of Molecular Toxicology, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden

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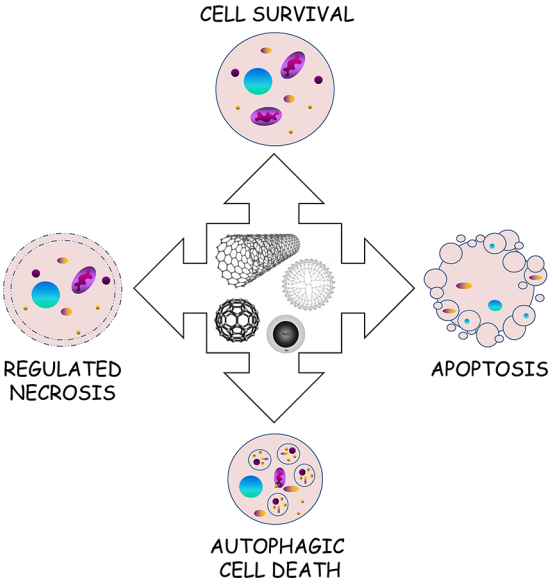
CONSPECTUS

Engineered nanomaterials offer numerous and tantalizing opportunities in many sectors of society, including medicine. Needless to say, attention should also be paid to the potential for unexpected hazardous effects of these novel materials. To date, much of the nanotoxicology literature has focused on the assessment of cell viability or cell death using primitive assays for the detection of plasma membrane integrity or mitochondrial function or assessment of cellular morphology. However, when assessing the cytotoxic effects of engineered nanomaterials, researchers need not only to consider whether cells are dead or alive but also to assess which of the numerous, highly specific pathways of cell death might be involved. Moreover, it is important to diagnose cell death based not only on morphological markers but on the assessment and quantification of biochemical alterations specific to each form of cell death.



In this Account, we provide a description of the three major forms of programmed cell death in mammalian cells: apoptosis, autophagic cell death, and regulated necrosis, sometimes referred to as necroptosis. Apoptosis can be activated via the extrinsic (death receptor-dependent) or via the intrinsic (mitochondria-dependent) route. Apoptotic cell death may or may not require the activation of cytosolic proteases known as caspases. Autophagy (self-eating) has an important homeostatic role in the cell, mediating the removal of dysfunctional or damaged organelles thereby allowing the recycling of cellular building blocks. However, unrestrained autophagy can kill cells. Studies in recent years have revealed that necrosis that depends on activation of the kinases RIP1 and RIP3 is a major form of programmed cell death with roles in development and immunity.

We also discuss recent examples of the impact of engineered nanoparticles on the three different pathways of programmed cell death. For example, acute exposure of cells to carbon nanotubes (CNTs) can induce apoptosis whereas chronic exposure to CNTs may yield an apoptosis-resistant and tumorigenic phenotype in lung epithelial cells. Several reports show that nanoparticles, including polystyrene particles, are routed to the lysosomal compartment and trigger cell death through the destabilization of lysosomal membranes with engagement of the intrinsic apoptosis pathway. In addition, a number of studies have demonstrated that nanomaterials such as CNTs, quantum dots, and gold nanoparticles can affect cellular autophagy. An improved understanding of the complexities of the nanomaterial-induced perturbation of different cell death pathways may allow for a better prediction of the consequences of human exposure.

Introduction

Cell culture (*in vitro*) studies are commonly applied to assess the impact of engineered nanomaterials.[1] It is frequently acknowledged that many of the commonly used cell viability tests to assess "cell death" are fraught with methodological problems related to interference of nanoparticles with the assay.[2] However, less attention is paid to the fact that cell death is no longer a black box; there are numerous, highly

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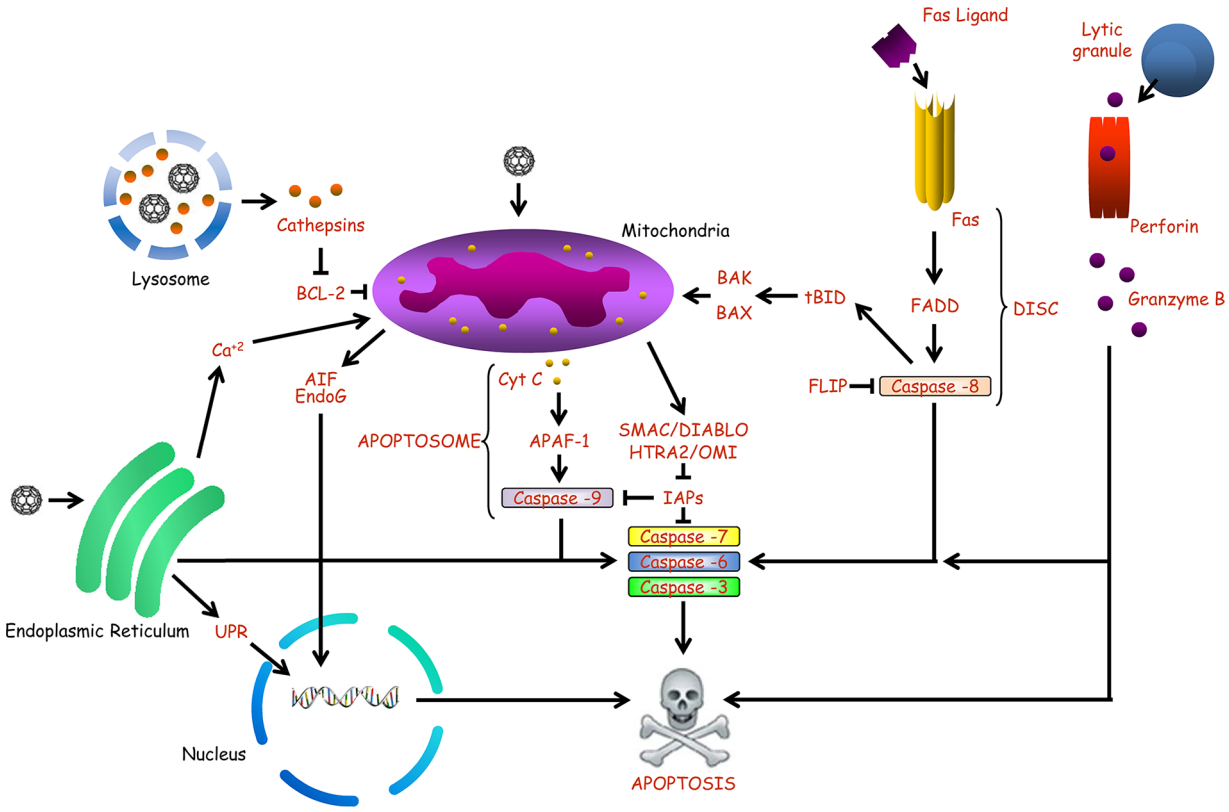

FIGURE 1. Programmed cell death: apoptosis. Schematic diagram depicting the two major pathways of apoptosis signaling in mammalian cells, the extrinsic or death receptor-mediated pathway and the intrinsic or mitochondria-dependent pathway.[5] Mitochondria act as integrators or amplifiers of various cell death stimuli. Nanomaterials, including single-walled carbon nanohorns,[40] TiO_2,[41] and polystyrene nanoparticles[44] may induce apoptosis through lysosomal impairment. PAMAMs have been shown to cause lysosomal[42] or mitochondrial membrane destabilization.[43] Silver[46] and gold nanoparticles[47] have been reported to induce ER stress, which may lead to cytochrome *c* release from mitochondria, thereby unleashing the apoptotic caspase cascade.

specific cell death modalities and most of the assays currently applied in nanotoxicological research will not capture the complexities of (programmed) cell death. It is our belief that the nanosafety research community would benefit greatly from applying a molecular diagnosis of cell death engaged by nanomaterials. Here, we describe the three major forms of programmed cell death and discuss recent studies linking nanoparticles to the regulation of different modes of cell death.

More than One Way To Skin a Cat

There has been a tendency among cell death researchers to dichotomize cell death into necrosis, which has been viewed as accidental, pathological cell death, and apoptosis, which is considered as genetically programmed and physiological in nature. However, in the most recent incarnation of the Nomenclature Committee on Cell Death set of recommendations, at least 13 different types of cell death are enumerated, and a systematic classification of cell death based on biochemical and functional considerations is presented.[3] Such complexity may seem daunting at first, but it would be a mistake to oversimplify; once we understand the specific signaling pathways underlying cell death in disease or in response to xenogenous agents, for example, nanoparticles, we may devise specific strategies to regulate or mitigate such effects. Oliver Wendell Holmes, Sr., the celebrated poet–physician, once declared "I would not give a fig for the simplicity this side of complexity, but I would give my life for the simplicity on the other side of complexity." Thus, we must learn to live with complexity, in order to understand how to prevent adverse effects of engineered nanomaterials and to enable the development of nanotechnologies that are safe-by-design.

There are three major forms of programmed cell death: apoptosis, autophagic cell death, and regulated necrosis (sometimes referred to as necroptosis). The term "programmed" in this context implies that the dismantling of the cell is regulated by specific genes and involves the activation of specific molecular pathways. The pathways regulating programmed cell death are, to a large extent, conserved through evolution.

Programmed Cell Death: Apoptosis

Apoptosis is a form of cellular suicide that can be divided (in mammalian cells) into *extrinsic* (dependent on so-called death receptors expressed in the plasma membrane) and *intrinsic* (dependent on/convergent on mitochondria-controlled signaling).[4] The extrinsic apoptosis pathway is by definition caspase-dependent, whereas the intrinsic apoptosis pathway may transpire by either caspase-dependent or caspase-independent signaling. The caspases are a family of intracellular cysteine-dependent, aspartate-specific proteases, which reside as latent precursors in most cells and propagate cell death (apoptosis) as well as differentiation/proliferation and inflammation. Extrinsic apoptosis is mediated via specific transmembrane receptors belonging to the tumor necrosis factor (TNF) receptor superfamily. Hence, ligands such as Fas ligand, TNF-α, or TRAIL (TNF-related apoptosis-inducing ligand) bind to death receptors, that is, Fas (also known as APO-1 or CD95), TNF-receptor I, and TRAIL receptor 1 or 2, resulting in oligomerization of the receptor at the cell surface and initiation of a signaling cascade in the cell culminating in apoptotic cell death.[5]

The ligation of Fas on susceptible target cells leads to the assembly of a multiprotein complex at the plasma membrane (Figure 1). The adaptor protein, FADD (Fas-associated death domain-containing protein) binds to Fas via its so-called death domain, a conserved, cytoplasmic sequence that is shared by all death receptors. This leads, in turn, to the recruitment of pro-caspase-8 (and pro-caspase-10). The resulting complex is termed the death-inducing signaling complex (DISC) and serves as a platform for caspase activation. In some cell types, referred to as type I cells the autocatalytic activation of caspase-8 at the DISC directly leads to proteolysis of pro-caspase-3 into active caspase-3, resulting in apoptosis independently of mitochondria. In other cells, so-called type II cells, caspase-8 mediates the cleavage of the BH3-only protein, Bid, leading to the generation of a mitochondria-targeted form known as truncated Bid or tBid and subsequent amplification of the death signal via mitochondria. Several other proteins modulate extrinsic apoptosis, such as cFLIP (cellular FLICE-like inhibitory protein) and the cIAPs (cellular inhibitor of apoptosis proteins).

Cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells are equipped with several proapoptotic weapons to destroy virus-infected or malignant cells.[4] Thus, in addition to the death receptor pathway, these cells also release cytotoxic proteins including the pore-forming protein perforin and several granzymes from so-called lytic granules. Upon entry into target cells, granzymes may activate caspase-dependent or -independent apoptosis (Figure 1). Viruses, in turn, have evolved strategies to circumvent cellular apoptosis, including viral FLIPs and several forms of caspase inhibitors such as crmA (poxvirus) and p35 (baculovirus).

The intrinsic pathway of apoptosis is engaged in response to numerous types of cellular stress including DNA damage, oxidative stress, cytosolic calcium overload, endoplasmic reticulum (ER) stress as a function of the accumulation of unfolded proteins, etc. Of particular interest for nanoparticle-induced apoptosis is the fact that lysosomal disruption with release of lysosomal proteases (cathepsins) also engages the mitochondria-dependent pathway of apoptosis (Figure 1), in part through the proteolysis of Bid.[6]

The intrinsic apoptosis pathway is characterized by multiple events: the dissipation of the mitochondrial transmembrane potential, the release of proapoptotic proteins into the cytosol, including cytochrome *c*, apoptosis-inducing factor (AIF), endonuclease G (EndoG), second mitochondria-derived activator of caspases (Smac, also known as Diablo), and high temperature requirement protein A2 (HtrA2, also known as Omi), and inhibition of the respiratory chain promoting overproduction of reactive oxygen species.[5] Pro- and antiapoptotic members of the Bcl-2 family modulate apoptosis through regulation of the release of mitochondrial factors including cytochrome *c*. The cytosolic translocation of cytochrome *c* triggers the formation of a multiprotein complex known as the apoptosome, consisting of cytochrome *c*, dATP, apoptotic protease-activating factor-1 (Apaf-1), and pro-caspase-9. This complex serves as a platform for caspase activation downstream of mitochondria (Figure 1). AIF and EndoG relocate from mitochondria to the nucleus where they mediate caspase-independent DNA fragmentation. HtrA2/Omi and Smac/Diablo block the activity of members of the IAP family, thereby derepressing caspase activation, leading to full-blown apoptosis.

Apoptosis plays a fundamental role in development and for maintenance of tissue homeostasis in the adult organism.[4] In addition, impairment of apoptosis may contribute to tumor progression. Conversely, numerous preclinical studies have demonstrated the therapeutic value of specific targeting of the apoptosis machinery, for example, IAPs and Bcl-2 family members in cancer cells,[7,8] and many clinical trials are underway.

Programmed Cell Death: Autophagy

Autophagy has an important homeostatic role, mediating the removal of dysfunctional or damaged organelles allowing the

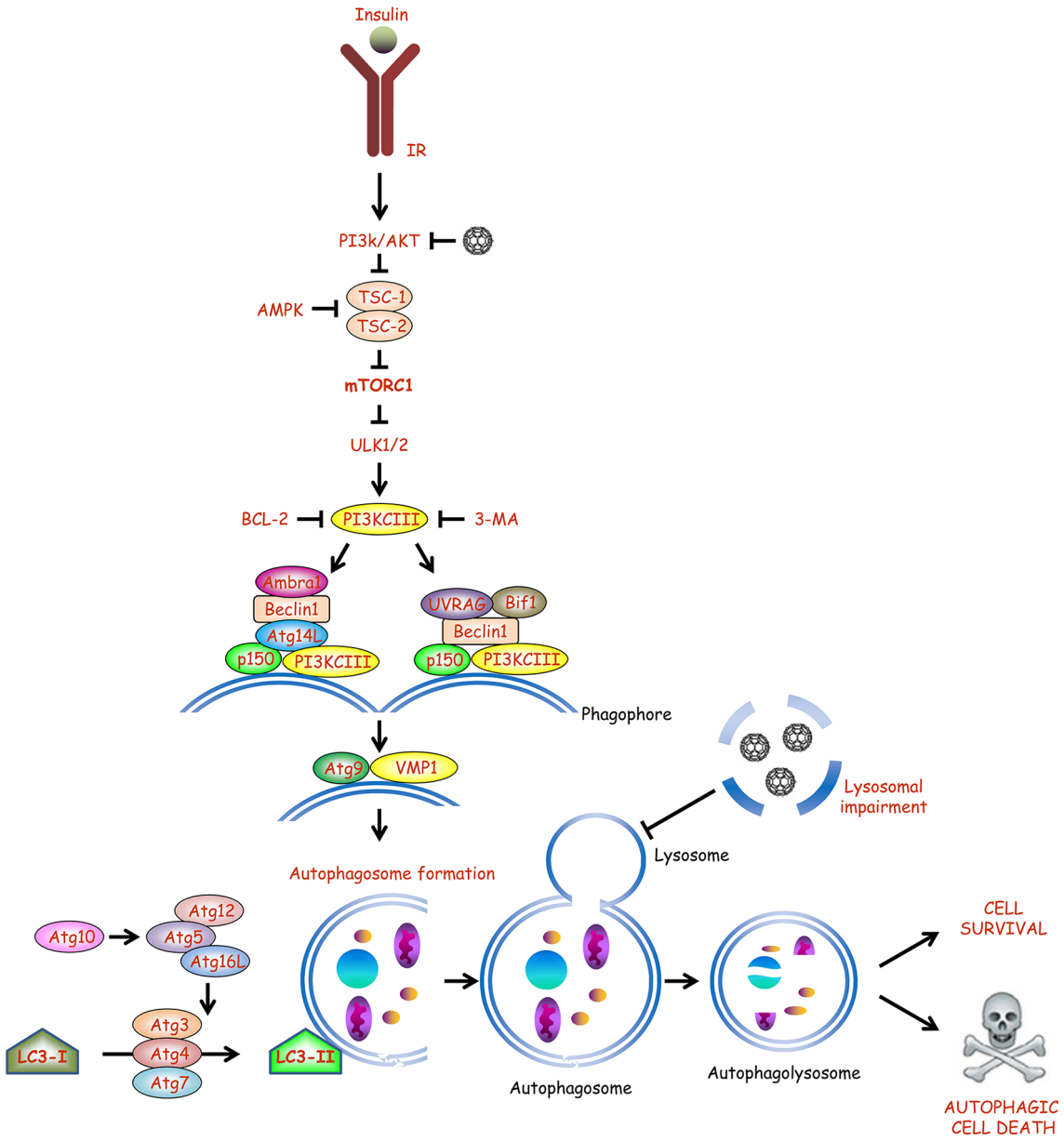

FIGURE 2. Programmed cell death: autophagic cell death. Autophagy (self-eating) is a survival mechanism deployed by cells to cope with conditions of nutrient deprivation.[9] However, unrestrained autophagy can result in genetically programmed cell death. Carbon nanotubes, PAMAMs, and iron oxide nanoparticles were reported to trigger autophagic cell death through the perturbation of the mTOR pathway,[53−55] while gold nanoparticles may induce autophagy blockade through lysosomal impairment.[56]

recycling of cellular building blocks.[9] In this sense, autophagy is a survival mechanism deployed by cells to cope with conditions of nutrient deprivation. However, unrestrained autophagy can result in cell death, and it is important to distinguish between "autophagy" and "autophagic cell death". Inhibition of cytoprotective autophagy would accelerate rather than prevent cell death.

The mammalian target of rapamycin complex 1 (mTORC1) acts as a major checkpoint in autophagy (Figure 2). Thus, mTORC1 integrates signaling through the PI3K/Akt pathway and cellular nutrient status or energy levels, which are sensed by AMP-activated kinase (AMPK). Autophagy is inhibited by signaling through growth factor receptors such as the insulin receptor that activate PI3K/Akt and promote mTORC1 activity through inhibition of TSC1/TSC2. Activated mTORC1 downregulates autophagy by phosphorylating a complex of autophagic proteins (ULK1/2). Conversely, autophagy is induced by nutrient starvation through the inhibition of mTORC1 leading to the recruitment of the class III phosphatidylinositol-3-OH kinase (PI3KCIII) complex with either

Beclin1 (coiled-coil myosin-like Bcl-2-interacting protein-1)−Atg14L−PI3KCIII−p150, Ambra1 (activating molecule in Beclin-1-regulated autophagy), or Beclin1−UVRAG−PI3KCIII−p150−Bif1 (Bax-interacting factor 1). Bcl-2 negatively regulates this step, thus demonstrating a degree of cross-talk between apoptosis and autophagy. The signaling culminates in the fusion of the double-membrane autophagosome with the lysosome into the autophagolysosome and the cargo-containing membrane compartment is then lysed and degraded.[9] Autophagy, in other words, may be viewed as genetically regulated "garbage recycling".

There is evidence of cross-talk between apoptosis and autophagy. Hence, Bcl-2 not only functions as an antiapoptotic protein but also as an antiautophagy protein via its inhibitory interaction with Beclin-1.[10] Moreover, a recent study has shown that a dynamic interaction exists between Ambra1 and Bcl-2 at the level of the mitochondrion that could regulate both Beclin-1-dependent autophagy and apoptosis.[11]

The autophagic machinery not only handles the lysosomal degradation of cellular building blocks but also orchestrates various responses to exogenous stimuli such as microorganisms.[12] For instance, autophagy plays a key role in the defense against bacterial infection.[13,14] Autophagy is also required for antigen presentation in an MHC class II-dependent manner, which is important for immune responses against viruses.[15]

The role of autophagy in cancer is complex: autophagy may allow cancer cells to overcome metabolic stress (e.g., hypoxia, nutrient deprivation), which could provide cancer cells with a survival advantage; however, autophagy may also counteract tumor development.[16] A recent study provided evidence that autophagy is dispensable for chemotherapy-induced cell death but is essential for the release of ATP from dying cells.[17]

Programmed Cell Death: Necrosis

Necrosis was thought for a long time to be merely an accidental form of cell death, but research from several laboratories in recent years has shown that necrosis can be regulated and may play a role in several pathological and physiological settings.[18] The term "necroptosis" is sometimes used as a synonym for regulated necrosis, but it was originally introduced by Yuan and co-workers to delineate a specific form of regulated necrosis that is triggered by death receptor ligation and blocked by necrostatin-1, a specific small-molecule inhibitor of necroptosis.[19] The authors subsequently demonstrated that the death domain receptor-associated adaptor, RIP1 kinase, acts as the cellular target of this novel class of antinecroptotic compounds.[20] It should be noted that RIP3-dependent, RIP1-independent cases of necrosis have been described, suggesting that there are several subprograms of regulated necrosis.[21]

Necrosis induced by death receptor (e.g., TNFR1) stimulation depends on the kinase activity of receptor interacting protein (RIP) 1 and 3. RIPK1 and RIPK3 are present with FADD, caspase-8, and possibly TRADD (TNF receptor-associated death domain-containing protein) in the so-called necrosome, which can induce apoptosis or necroptosis (Figure 3). Normally, as discussed above, caspase-8 activation triggers apoptosis, but if this caspase is absent or blocked, RIPK1 and RIPK3 become phosphorylated, and this leads to regulated necrosis. Regulated necrosis can also be induced by alkylating DNA damage (possibly by the overactivation of poly(ADP-ribose) polymerase 1, PARP1). Furthermore, cells recognize pathogens upon binding of pathogen-associated molecular patterns (PAMPs) to toll-like receptors (TLRs), and this induces RIPK1- and RIPK3-dependent necrosis, involving the TIR-related adaptor protein inducing IFN (TRIF). Upon initiation of necrosis, several factors are involved in its conditioning and execution, including calcium-activated calpain activation that results in lysosomal membrane permeabilization, sphingomyelinase-mediated ceramide generation, and activation of Jun N-terminal kinase (JNK) (Figure 3).

In a genome-wide siRNA screen, Hitomi et al.[22] defined a signaling network that regulates necroptosis and elucidated the interconnectedness between apoptosis and necroptosis. For instance, the BH3-only protein Bmf, previously implicated in apoptosis, was identified as a core component in necroptotic cell death signaling. Recent studies provide further evidence that the different pathways of cell death are closely intertwined. Hence, the adapter protein FADD, the death-executing caspase-8, and cFLIP, a regulator of caspase-8 activity, were shown to inhibit RIPK1- and RIPK3-dependent necroptosis during development, in particular of the immune system.[23−25]

Necroptosis has been implicated in neuronal excitotoxicity, which is linked to neurological disorders such as Parkinson's disease, Huntington's disease, and Alzheimer's disease, and contributes to ischemic brain injury in mice.[18] In addition, RIP3-deficient mice exhibit impaired virus-induced tissue necrosis, inflammation, and control of viral replication suggesting that RIP3-dependent necrosis is necessary for the response against virus infections.[26] Recently, a cytomegalovirus-encoded protein inhibitor of RIP1 designated M45 was discovered that can block RIP1 signaling thus providing further evidence that necroptosis is involved in the immune response to viruses.[27]

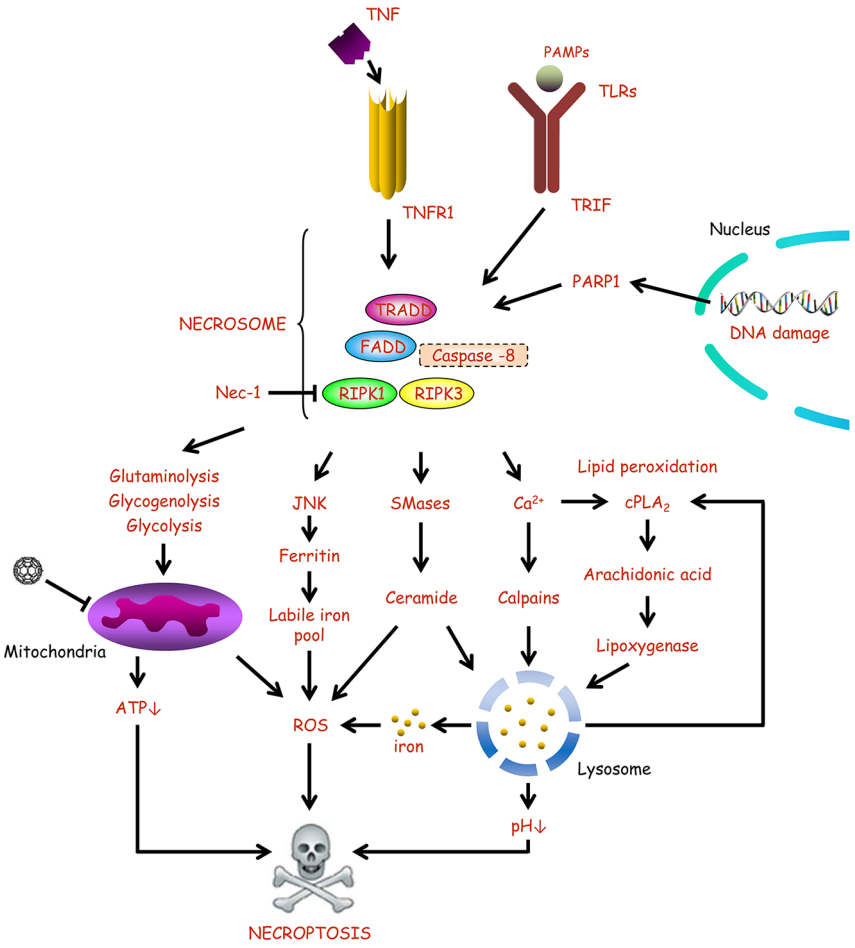

FIGURE 3. Programmed cell death: regulated necrosis. During regulated necrosis, different stimuli are recognized or sensed by specific receptors on the cell surface or inside cells.[18] The activation of the necrosome stimulates different signaling pathways leading to mitochondrial hyperpolarization, lysosomal membrane permeabilization, and generation of reactive oxygen species (ROS), leading to cellular disintegration. A recent study revealed that germanium nanoparticles trigger necrostatin-1-inhibitable cell death with a reduction of the mitochondrial membrane potential.[58]

The Importance of Being Small

Orrenius et al. recently discussed the implications of different cell death mechanisms in toxicology (of chemicals, drugs, environmental pollutants).[28] Similarly, we posit that an increased understanding of the complexities of nanomaterial-induced perturbation of different cell death pathways will allow for a better prediction of the consequences of human exposure to these materials. One may ask whether there are any examples of nanospecific effects or do nanomaterials merely activate a final common pathway of cellular demise irrespective of size or other specific physicochemical properties? A pertinent illustration of nanomaterial-specific effects, that is, effects specifically related to the smallness of the materials in question, is the systematic study by Pan et al.[29] of gold nanoparticles ranging in size from 0.8 to 15 nm, in which the 1.4 nm gold particles were found to be the most cytotoxic and the cellular response was found to be size-dependent in that 1.4 nm particles caused necrosis while the 1.2 nm particles predominantly induced apoptosis. The 15 nm particles were nontoxic. Furthermore, Kim et al.[30] reported that small (10 nm) silver nanoparticles had a greater ability to induce apoptosis than other-sized silver nanoparticles (50 and 100 nm). It is well established that the mode of cell death depends on the severity of the cellular insult, which may, in turn, be linked to mitochondrial function and intracellular energy. Foldbjerg et al.[31] reported that silver nanoparticles induced apoptotic and necrotic cell death in a dose- and time-dependent manner; however, one should bear in mind that the induction of apoptosis in cell culture is inevitably followed by secondary necrosis. Moreover, other nanomaterial physicochemical properties such as size, shape, and surface charge also come into play. Shaeublin et al.[32] reported that charged gold nanoparticles induced cell death through apoptosis whereas neutral gold nanoparticles triggered necrosis in a human keratinocyte cell line. The mode of cell death induced by nanoparticles may also be cell

type-specific[30,33] (and see below). Finally, the efficiency of cellular uptake of nanoparticles and the resultant intracellular dose may determine the cytotoxic potential.[34] However, nanoparticles may also induce apoptosis in individual cells that then propagates to other neighboring cells.[35]

Effects of Nanoparticles on Apoptosis

A number of studies have been published on the effect of carbon nanotubes (CNTs) on apoptosis. MWCNTs were reported to induce apoptosis in A549 lung carcinoma cells at doses of 10 and 50 μg/mL, as estimated by nuclear condensation and DNA laddering, but the underlying mechanism was not disclosed.[36] Thurnherr et al.,[37] on the other hand, did not observe overt cell death in A549 cells and Jurkat T cells following acute exposure to MWCNTs up to 30 μg/mL and noted that the continuous presence of low amounts of MWCNTs (0.5 μg/mL) for 6 months did not induce cell death even as large amounts of the nanotubes accumulated in A549 cells. Wang et al.[38] developed a chronic exposure model in which human lung epithelial cells were continuously exposed to very low amounts (0.02 μg/mL) of SWCNTs. After 6 months, the cells displayed a significant increase in cell proliferation. Most importantly, the long-term exposed cells were apoptosis-resistant and induced tumor formation *in vivo*. Using a global oxidative lipidomics approach, Tyurina et al.[39] revealed a highly selective pattern of pulmonary lipid peroxidation after exposure of mice to SWCNTs and a concomitant increase in apoptotic neutrophils in the lungs. Thus, peroxidation was confined to three relatively minor classes of phospholipids including mitochondria-specific cardiolipin (CL). A similar phospholipid peroxidation profile is seen in apoptotic cells. These results suggest that the *in vivo* exposure to SWCNTs leads to the activation of specific apoptosis signaling pathways and indicates that agents that protect against selective lipid peroxidation could prevent the deleterious effects of CNTs.[39]

Nanoparticles are frequently detected in lysosomes upon internalization. Indeed, in a recent study, single-walled carbon nanohorns were shown to undergo uptake in RAW 264.7 murine macrophages, and the nanoparticles preferentially localized to lysosomes,[40] resulting in destabilization of lysosomal membranes leading to apoptotic, as well as necrotic, cell death. Hussain et al.[41] found that carbon black nanoparticles (13 nm) induced apoptosis in bronchial epithelial cells via intrinsic apoptosis signaling with activation of Bax and release of cytochrome *c* from mitochondria whereas TiO_2 nanoparticles (15 nm) induced apoptosis through lysosomal membrane destabilization and cathepsin B release, suggesting that the pathway of apoptosis varies depending on the chemical nature of the nanoparticles. Poly(amidoamine) (PAMAM) dendrimers were found to be taken up into the lysosomal compartment in KB cells, a subline of HeLa cells, and they increased lysosomal pH and triggered apoptosis as a function of the number of surface amino groups.[42] Other investigators have shown that PAMAM dendrimers colocalized with mitochondria in human lung cells *in vitro* and caused the release of cytochrome *c* and caspase activation.[43] Nel and colleagues showed that cationic polystyrene nanoparticles (60 nm) entered the lysosomal compartment in RAW 264.7 cells from where the particles could escape by lysosomal rupture.[44] The release of particles into the cytosol induced an increase in mitochondrial Ca^{2+} uptake and cell death that could be suppressed by cyclosporin A, an inhibitor of permeability transition pore opening, a key step in intrinsic apoptosis. Similar cytotoxic effects were seen in epithelial BEAS-2B cells, but not in microvascular endothelial cells, thus demonstrating cell-specific sensitivity or resistance to nanoparticle-induced apoptosis.[44]

In an *in vitro* study designed to address the potential health effects of airborne nanoparticles, we showed that palladium nanoparticles (10 nm) induced cell death in human primary bronchial epithelial cells (PBEC) but not in the A549 cell line, at the doses tested (up to 25 μg/mL).[45] The activation of caspase-3 in PBECs was detected using a specific fluorescent peptide substrate and Western blotting to detect the active fragment of caspase-3. The A549 cell line is commonly used in toxicology, but these cells are notoriously apoptosis-resistant. Our unpublished data reveal that the palladium nanoparticles are able to trigger cell death in other cancer cells at higher doses (>50 μg/mL).

Overall, it appears that nanoparticle-triggered apoptosis commonly occurs through endosomal uptake of particles and translocation to the lysosomal compartment, followed by lysosomal destabilization and release of cathepsins that activate the mitochondria-dependent (intrinsic) pathway of apoptosis (Figure 1). Zhang et al.[46] reported that silver nanoparticles may exert cytotoxic effects through modulation of ER stress; this could also, in turn, lead to activation of mitochondria-dependent apoptosis. Moreover, using systems biology approaches, Tsai et al.[47] provided evidence for gold nanoparticle-induced apoptosis in K562 leukemia cells through induction of unmanageable ER stress, leading to mitochondrial cytochrome *c* release, but insignificant toxicity to peripheral blood mononuclear cells, the normal counterpart of K562 cells.

Effects of Nanoparticles on Autophagy

Several studies have suggested that nanoparticles may function as autophagy activators. Early work by Seleverstov et al.[48] showed that quantum dots induce autophagy in human mesenchymal stem cells in a size-dependent manner. Similarly, Stern et al.[49] provided evidence that CdSe quantum dots were cytotoxic for porcine kidney cells and detected autophagy based on ultrastructural changes and LC3 immunoblotting. Several other classes of nanoparticles including fullerenes, gold nanoparticles, iron core–gold shell nanoparticles, and iron oxide nanoparticles have been shown to activate autophagy *in vitro* or to induce cell death or growth inhibition with concomitant formation of autophagic vacuoles.[50−52] The question is whether this is a defense mechanism in cells that have taken up nanosized particles or whether autophagic cell death is responsible for the cytotoxicity of the nanoparticles (Figure 2). Khan et al.[53] reported that iron oxide nanoparticles (50 nm) trigger autophagic cell death in human lung cancer cells with involvement of the classical mTOR pathway. More recently, *in vivo* studies have provided evidence for a pathological role of autophagy. Hence, PAMAM dendrimer-induced cytotoxic effects were found to be mediated via autophagic cell death because the autophagy inhibitor 3-methyladenine (3-MA) ameliorated acute lung injury in mice and 3-MA treatment or silencing of Beclin-1 rescued A549 cells from cell death.[54] Moreover, carboxylic acid-functionalized SWCNTs also induce the formation of autophagosomes in A549 cells and 3-MA reduced COOH-SWCNT-induced cell death and reduced the acute lung edema in mice.[55]

It is important, however, to recognize that methods for detection of an increase in autophagy over baseline need to take into account the dynamic nature of the process.[3] That is to say, steady-state methods do not provide a reliable estimate of autophagic activity because they do not discriminate between enhanced rates of autophagy (increased on-rate) and inhibition of autophagosomal–lysosomal fusion (decreased off-rate). Ma et al.[56] reported that gold nanoparticles (10−50 nm) induced autophagosome accumulation through size-dependent uptake and lysosomal impairment. However, the authors noted that autophagosome accumulation resulted from a blockade of autophagy flux rather than induction of autophagy (Figure 2). As pointed out by the authors, autophagy induction and basal autophagy blockade have opposite effects on intracellular degradation: the former enhances degradation, while the latter inhibits it.[56] Hence, certain nanoparticles may essentially lead to "lysosomal storage disease" at the cellular level through their interference with lysosomal function.

Effects of Nanoparticles on Necroptosis

There are scattered examples in the literature of other forms of nanoparticle-triggered cell death. For instance, 10 nm carbon black nanoparticles were shown to trigger pyroptosis, a pro-inflammatory, caspase-1-dependent form of cell death seen in macrophages.[57] In addition, in a recent study, 4 nm germanium nanoparticles were found to induce cell death, as evidenced by the MTT test, and this was subsequently shown to be inhibitable by necrostatin-1 (Figure 3), suggesting that this is the first example of nanoparticle-induced necroptosis or regulated necrosis.[58] The challenge in studying necroptosis is that there are as yet no single discriminative biochemical markers available.[18]

Conclusions and Perspectives

In this Account, we have attempted to emphasize the importance of not only considering whether cells are dead or alive but also assessing different pathways of cell death when assessing the cytotoxic effects of engineered nanomaterials. There are examples in the literature of nanoparticle effects on programmed cell death, but the overwhelming majority of nanotoxicological studies fail to distinguish among different cell death modalities, and important information is therefore lost. One reason for this may be the choice of methods used to detect nanoparticle-induced cytotoxicity. Notwithstanding, based on the available data, it appears that lysosomes, dubbed "suicide bags" by the Nobel laureate Christian de Duve who discovered these organelles, may play a special role in cellular responses to nanoparticles, and it is certainly worth noting that lysosomes may participate not only in apoptosis but also in autophagy/autophagic cell death and in the amplification of regulated necrosis.

Resistance to apoptosis is one of the hallmarks of cancer.[59] Therefore, it is prudent to carefully consider the use of cancer cell lines for the study of nanoparticle-triggered apoptosis as well as other modes of programmed cell death; a model is only a model, and studies using transformed cell lines should be complemented with studies of primary cells or studies using more advanced *in vitro* and *in vivo* (animal) model systems.[60]

In synopsis, the many different pathways of programmed cell death offer numerous targets for engineered nanomaterials. Understanding the mode of action of nanomaterial-induced cytotoxicity may lead to more refined approaches for the mitigation of adverse effects of these materials and

to a more reliable risk assessment of their effects on human health. In addition, insights regarding nanomaterial-induced perturbation of cell death pathways may also be of relevance for biomedical applications of nanomaterials and could be harnessed for therapeutic purposes.

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BIOGRAPHICAL INFORMATION

Fernando Torres Andón obtained a Ph.D. in Molecular Biology from the University of Las Palmas de Gran Canaria in 2010 and is currently a postdoctoral researcher at Karolinska Institutet, Stockholm. His research is focused on the assessment of hazardous effects of carbonaceous nanomaterials on the immune system.

Bengt Fadeel, M.D., Ph.D., is Head of the Division of Molecular Toxicology and Vice Chairman of the Institute of Environmental Medicine at Karolinska Institutet in Stockholm. He is Professor of Medical Inflammation Research at Karolinska Institutet and Adjunct Professor of Environmental and Occupational Health at the University of Pittsburgh, Pittsburgh, PA. He coordinated FP7-NANOMMUNE, an EU−US consortium focused on the assessment of hazardous effects of nanomaterials on the immune system, and he is the theme leader of nanomaterial hazard activities in FP7-MARINA, a project dealing with reference methods for risk management of nanomaterials.

FOOTNOTES

*Corresponding author. Mailing address: Division of Molecular Toxicology, Institute of Environmental Medicine, Nobels väg 13, Karolinska Institutet, 171 77 Stockholm, Sweden. Tel: +46 8 524 877 37. Fax: +46 34 38 49. E-mail: bengt.fadeel@ki.se.
The authors declare no competing financial interest.

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