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Regulated cell death in diagnostic histopathology

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ABSTRACT Regulated cell death (RCD) is a controlled cellular process, essential for normal development, tissue integrity and homeostasis, and its dysregulation has been implicated in the pathogenesis of various conditions including developmental and immunological disorders, neurodegenerative diseases, and cancer. In this review, we briefly discuss the historical perspective and conceptual development of RCD, we overview recent classifications and some of the key players in RCD; finally we focus on current applications of RCD in diagnostic histopathology.

KEY WORDS: *regulated cell death, programmed cell death, apoptosis, histopathology, diagnostic markers*

Introduction

Regulated cell death (RCD) is a controlled cellular process occurring in physiological and pathological conditions. It is essential for normal development, tissue integrity and homeostasis in multicellular organisms. Dysregulation of cell death routines leads to a variety of pathological conditions, including developmental and immunological disorders, neurodegenerative diseases, and cancer (Fuchs and Steller, 2011). In this review, following the recent recommendations of Nomenclature Committee on Cell Death (NCCD) (Galluzzi *et al*., 2014), we refer to the programmed cell death (PCD) in cases of physiological regulated cell death.

Historical aspects

The research field of cell death has been emerging since few decades ago. Though, the story of RCD begins as early as 1842, when Carl Vogt noticed dying cells in toad embryos (Vaux and Korsmeyer, 1999), and in 1885 when some of the distinct morphological aspects, as opposed to necrosis, were recognized and described as spontaneous cell death, named chromatolysis, by Walther Flemming (Majno and Joris, 1995). The discovery of tissue stains has enabled early researchers to encounter this phenomenon in many tissues, such as regressing follicles, lactating mammary glands or breast cancer (Majno and Joris, 1995), and eventually, the questions about the process opposite to mitosis and the regulation of tissue growth rose. In 1914 a German anatomist Ludwig Gräper gathered the existing knowledge and the results of his own experiments about the physiological elimination of cells, in an article that should had laid the foundations for the field, but unfortunately it did not attract much attention (Majno and Joris, 1995). Many years later more detailed descriptions and underlying mechanisms of programmed cell death were given by Glucksman in 1951, and Kerr, Lockshin and Williams in 1965 (Ameisen, 2002, Galluzzi *et al*., 2012, Kerr, 1965, Lockshin and Williams, 1965).

Kerr suggested two morphological types of cell death at that time, first being degenerative, essentially the necrosis, and the second, non-degenerative, which he had named "shrinkage necrosis" (Kerr, 1965, Kerr, 2002). Degenerative cell death was described as affecting groups of cells, including cell swelling and disruption of cell membranes, release of cytoplasm and inflammatory response. The non-degenerative cell death was affecting single or small clusters of cells, featured cell and nucleus shrinkage, preserved cell membrane, and no inflammatory cells were present. In the years that follow, shrinkage necrosis was observed in a wide range of physiological and pathological states, including embryogenesis, spermatogenesis, tissue remodeling during regression or healing, and cancer growth (O'Rourke and Ellem, 2000), eventually leading to concept of apoptosis (Kerr *et al*., 1972, Wyllie *et al*., 1980).

Apoptosis (*Greek*, falling of the leaves) was defined as an active, programmed and controlled cell deletion mechanism, which plays a complementary but opposite role to mitosis in regulation of cell population number, and can be triggered by both physiological and pathological stimuli (Kerr *et al*., 1972). The expansion of molecular biology tools has generated a new wave of research in this field, resulting in the identification of several specific genes and proteins

Abbreviations used in this paper: AI, apoptotic index; DLBCL, diffuse large B-cell lymphoma; GVHD, graft versus host disease; NCCD, Nomenclature Committee on Cell Death; PCD, programmed cell death; RCD, regulated cell death; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling.

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involved in apoptosis. The studies of the development of nematode *Caenorhabditis elegans* (Brenner, 2003, Horvitz, 1999, Sulston *et al.*, 1983) have greatly contributed to the understanding of the molecular basis of apoptosis and the authors were consequently awarded a Nobel Prize for Medicine in year 2002. Until recently apoptosis and PCD were used synonymously (Elmore, 2007).

Since 1994, the research in the field of cell death has greatly expanded (Fig. 1). The morphological characteristics of cell death were complemented by accumulating data on its biochemical and molecular properties. It became evident that RCD may not refer merely to apoptosis, but rather to a number of genetically regulated processes, resulting in one of the morphologically well-defined cell death types, namely apoptosis (Galluzzi *et al.*, 2012, Ouyang *et al.*, 2012), autophagic cell death (Shimizu *et al.*, 2014) or necroptosis (Kitanaka and Kuchino, 1999, Vanden Berghe *et al.*, 2014, Vandenabeele *et al.*, 2010, Zhou and Yuan, 2014) (Table 1). In addition, several novel, tentative modalities emerged, yet to be more characterized (Table 2). The most surprising fact discovered recently was that necrosis, historically considered as the accidental cell death type, may occur in finely regulated manner called *necroptosis* (Kaczmarek *et al.*, 2013).

Regulated cell death modalities

Rapid development and recognition of different types of cell death modalities were accompanied by inconsistent terminology used by various scientists and a need for clear definitions for the proper classification of cell death became evident. Initially, three types of cell death were proposed based on morphology (Schweichel and Merker, 1973). Proposed Type I, II and III of cell death were consistent with present concepts of apoptosis, autophagy, and necrosis, respectively (Kroemer *et al.*, 2005). This classification has been longstanding, and due to the simplicity and availability of morphological versus biochemical methods of RCD detection, even today it is used in its modified form (Shimizu *et al.*, 2014) (Table 1). In the meantime, many aspects of the process leading to cell death have been elucidated. Accordingly, cell death could be classified using different approaches including enzymological (involvement of nucleases and proteases), functional (programmed or accidental, pathological or physiological), immunological (immunogenic or non-immunogenic) (Galluzzi *et al.*, 2007), and finally molecular criteria (Galluzzi *et al.*, 2012). The Nomenclature Committee on Cell Death (NCCD) was formed in 2005 with the aim to propose unified criteria for the definition of cell death and different cell death morphologies (Kroemer *et al.*, 2005).

NCCD has, up to date, released four sets of recommendations on classification of cell death, the first one published in 2005 (Kroemer *et al.*, 2005), second in 2009 (Kroemer *et al.*, 2009), third in 2012 (Galluzzi *et al.*, 2012) and fourth in 2014 (Galluzzi *et al.*, 2014). In the first round of recommendations the classification relied purely on morphological criteria (Galluzzi *et al.*, 2007), and although many different underlying molecular mechanisms of cell death were known, available data was still not sufficient to clarify morphological and molecular correlations to overcome the widespread morphological classification. Subsequent NCCD classifications recommend accurate molecular definitions and precise molecular methods for detecting and quantifying the cell death (Galluzzi *et al.*, 2012, Kroemer *et al.*, 2009), moving towards less subjective interpretations and better reproducibility of the research results. In their most recent recommendations, NCCD proposes fundamentally two types of cell death: the accidental cell death (ACD) and the regulated cell death (RCD). The ACD occurs in an uncontrollable manner due to the extreme physical, chemical or mechanical stimuli followed by structural disintegration of the cell. The RCD is genetically regulated in both pathological and physiological conditions. When occurring in physiological conditions, the RCD is referred as programmed cell death (PCD) (Galluzzi *et al.*, 2014).

Recent studies revealed that morphologically and biochemically distinct RCD subroutines are not linear. They are not confined to single molecular pathway, nor mutually exclusive, but rather a complex interaction and crosstalk exists between them (Amaral *et al.*, 2010, Galluzzi *et al.*, 2012, Hotchkiss *et al.*, 2009). If single RCD pathway is defective or blocked the cell may continue by alternative one, such in a case of inhibition of apoptosis with caspase inhibitors, when cells may die by necroptosis (Degterev *et al.*, 2005, Ondrouskova *et al.*, 2008, Vandenabeele *et al.*, 2006). A multitude of different factors including stress signal type and intensity, cell

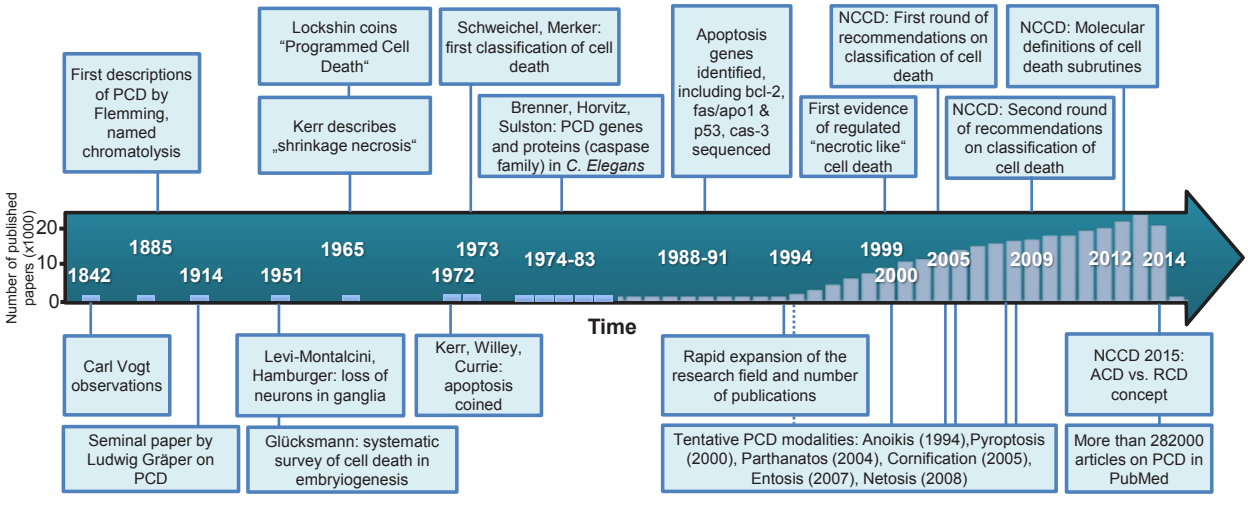

Fig. 1. The timeline of the progress of cell death research. *PCD, Programmed cell death; NCCD, Nomenclature Committee on Cell Death; ACD, Accidental cell death; RCD, Regulated cell death.*

type, and more to be discovered, may activate single or multiple RCD pathways, with complex interactions between pro- and anti-apoptotic signals, and overlapping; determining whether and by which RCD pathway the cell will end its life (Galluzzi *et al.*, 2012, Haupt *et al.*, 2003).

The important role of p53 protein and Bcl-2 protein family in regulated cell death

The p53 protein is a tumor suppressor and transcriptional factor, and is situated at a crossroad of a signaling pathway network, which determines the fate of a stressed cell (Levine and Oren, 2009). It acts as a switch for transcription of sets of genes to perform the temporary cell cycle arrest and DNA repair, or permanent growth arrest and senescence, or apoptosis. The ability of p53 to induce the senescence or apoptosis represents its crucial tumor suppressor function (Pietsch *et al.*, 2008), and it is not surprising that one of the most commonly mutated genes in cancer is the *TP53,* gene coding for p53 protein (Kumar *et al.*, 2013). Owing to its roles, the inactive or missing p53, due to mutations, deletions, degradation or viral inhibition, leads to a wide instability of the genome, eventually resulting in malignant transformation of the cell. Recent studies report that the TP family members, namely TP63 and TP73 may contribute to, and in some instances induce the apoptosis (Pietsch *et al.*, 2008)

p53 stimulates an extensive network of signals that act through both intrinsic and extrinsic apoptotic pathways. The cell fate in intrinsic (mitochondrial) apoptotic pathway depends on predominance of pro-apoptotic Bax and BH3 subfamily proteins (Bax, Bak, Noxa, and Puma (Amaral *et al.*, 2010)) or anti-apoptotic (Bcl-2, Bcl-X_L, and other (Reed, 1997)) signaling proteins of Bcl-2 family, which converge on mitochondrial membrane. Upon cell stress p53 mediates transactivation of various pro-apoptotic Bcl-2 members or represses some of the anti-apoptotic genes (e.g., *survivin, bcl-2*) (Hoffman *et al.*, 2002), or may post-translationally bind to Bcl-2 protein and inactivate it (Hemann and Lowe, 2006), which results in execution of intrinsic apoptosis. Modulation of extrinsic (death and dependence receptor) apoptotic pathway by p53 may occur by enhancing mRNA expression or protein trafficking of FAS receptor, which is tissue specific, or transactivation of others (e.g., DR5) (Haupt *et al.*, 2003).

Regulated cell death in normal development, homeostasis and disease

Normal development and differentiation require well-regulated orchestration of programmed cell division and cell death. Apoptosis is the most common cell death program in the developing embryo and occurs in many organs, including brain, liver, eye, the lymphoid system, and limb. PCD has important role in organ and

TABLE 1

MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF CELL DEATH MODALITIES

	Cell Death modality	Biochemical characteristics	Morphological characteristics
Regulated cell death*	**Apoptosis** (Galluzzi *et al.*, 2012)		
	Intrinsic	Induced by various intracellular stress conditions (DNA damage, hypoxia, high concentrations of Ca^{2+}, severe oxidative stress). Modulated by Bcl-2 family of pro- and anti-apoptotic proteins converging to mitochondrial membrane	Affects single cells or small clusters of cells. Characterized by: • rounding-up of cells • reduction of cell volume (pyknosis) • chromatin condensation • nuclear fragmentation (karyorrhexis) • little or no modification of organelles • plasma membrane blebbing but integrity until final steps of the process • formation of apoptotic bodies • engulfment of apoptotic bodies by surrounding cells and professional phagocytes • in most instances no inflammation present
	Caspase dependent	Executed by MOMP → release of IMS proteins (CYTC, Smac/DIABLO) → apoptosome formation → caspase-9 → caspase-3 pathway.	
	Caspase independent	Executed by MOMP → release of IMS proteins (AIF, ENDOG, HTRA2) → large-scale DNA fragmentation, cleaving of cytosolic and cytoskeletal proteins	
	Extrinsic	Caspase-dependent	
	By death receptors	Induced by binding of the death ligands (FASL/CD95L, TNFα, TRAIL) to transmembrane death receptors (FAS,TNFR1, TRAILR, DR5, other), executed by death receptor signaling (DICS) and - caspase 8 (or -10) → caspase-3 pathway; or - caspase-8 → tBID → MOMP → caspase-9 → caspase-3 pathway.	
	By dependence receptors	Induced by deprivation of ligands (e.g. NETRIN) binding to transmembrane dependence receptors (e.g DCC, UNC5B). Executed by dependence receptor signaling and direct or MOMP-dependent activation of caspase-9 → caspase-3 pathway.	
	Autophagic cell death (Green *et al.*, 2011, Liu and Levine, 2014)	Distinct molecular pathway, involving autophagy modulator genes (e.g., VPS34, AMBRA1, ATG5, ATG12, BCN1). May be induced by nutrient starvation, DNA damage, organelle damage, etc. Cytoplasmic organelles or protein aggregates are sequestered in double membrane vesicles and delivered to lysosomes for degradation. In stress-induced cases has *cytoprotective role and favors cell survival.* In normal development favors cell death.	Characterized by • massive vacuolization of the cytoplasm • lack of chromatin condensation • plasma membrane rupture • no engulfment by adjacent cells • usually no inflammation present
	Necroptosis (Kitanaka and Kuchino, 1999, Vanden Berghe *et al.*, 2014, Zhou and Yuan, 2014)	Induced by various stimuli including DNA damage, excitotoxins, ligation of death receptors (e.g. TNFR1) or apoptosis deficient conditions. Regulated by RIP1 and RIP3.	Affects large groups of cells or parts of tissue. Characterized by: • gain of cell volume (oncosis) • swelling of organelles • karyolysis, karyorrhexis, pyknosis • disrupted plasma membrane • leakage of intracellular contents • inflammation present
Accidental cell death	**Necrosis** (Elmore, 2007)	Induced by harsh external noxious stimuli, such as acute injury or infection, not showing biochemical or morphological features of apoptosis or autophagy.	

*Regulated cell death occurring in purely physiologic conditions is referred as Programmed cell death (PCD). MOMP, mitochondrial outer membrane permeabilization; IMS, inter membrane space; AIF, apoptosis inducing factor; ENDOG, endonuclease G; TNFα, Tumor Necrosis Factor alpha; TRAIL, TNF-related apoptosis inducing ligand; RIP1, Receptor interacting protein 1; RIP3, Receptor interacting protein 3; Smac/DIABLO, second mitochondria-derived activator of caspase/direct IAP binding protein with low Pi; CYTC, Cytochrome C; TNFR1, Tumor necrosis factor receptor 1; DCC, Deleted in Colorectal Carcinoma; DISC, Death Inducing Signaling Complex

tissue remodeling, the formation of hollow organs and the neural tube, and the generation of sexual organs (Jacobson *et al.*, 1997), shaping forms (e.g. interdigital clefts) and separating, splitting or allowing tissue layers to fuse (Entezari *et al.*, 2010, Penaloza *et al.*, 2006). During development, PCD is particularly prominent in central nervous system and the immunogenic cells of the hematopoietic system. Both of these systems develop through overproduction of the cells. In the central nervous system, two- or three-fold number of overproduced neurons and glial cells are reduced through the death of those neurons that do not have appropriate synaptic connections (Nijhawan *et al.*, 2000). In the immune system antigen-specific receptors are generated by random rearrangement of related structural genes and many potentially dangerous or useless immune cells are produced. Autoreactive cells are eliminated by overstimulation of death receptors, while useless cells, which lack appropriate receptor-ligand interactions or are deprived of stimulating factors, such as interleukin 2 (IL-2), are eliminated by dependence receptor apoptotic pathway (Opferman, 2008).

PCD is underlying the involution of hormone-dependent tissues upon hormone deprivation, such as endometrial cell breakdown during the menstrual cycle (Fig. 2A), and regression of the lactating breast after weaning; follicular atresia of postovulatory follicle; regulation of cell number in proliferating cell populations, such as intestinal crypt epithelia; elimination of cells that have served their function, as in a case of neutrophils in acute inflammation and lymphocytes at the end of immune response (Kumar *et al.*, 2013). In addition, cell death machinery is used by cytotoxic T lymphocytes for killing of the pathogen-invaded cells (Vince and Silke, 2009).

Imbalances in apoptosis are underlying or accompanying number of pathologic conditions, and importantly, resistance to cell death is recognized as one of the hallmarks of cancer (Elmore, 2007, Hanahan and Weinberg, 2011) (Fig. 2E-G). Dysregulation may occur at any of the phases of cell death program through inhibition or overactivation of death receptors and ligands, mutations in p53, overexpression, inhibition or mutation of Bcl-2 pro- and anti-apoptotic proteins, reduced expression of caspases or overexpression of inhibitors of apoptosis (IAPs)(Vince and Silke, 2009, Wong, 2011).

Failure of differentiation between adjacent digits caused by the absence of apoptosis in the interdigital mesenchyme results in syndactyly, a congenital condition characterized by the fusion of adjacent fingers (Jordan *et al.*, 2012). Exposure of intrauterine developing human embryo to noxious stimuli may cause cell stress and inappropriate or excess apoptosis, resulting in teratogenic effects (Brill *et al.*, 1999, Entezari *et al.*, 2010).

Increased apoptosis rate has been described in neurodegenerative diseases, such as Alzheimer's disease (Rohn, 2010), Parkinson's disease (Simunovic *et al.*, 2009), amyotrophic lateral sclerosis (Pasinelli *et al.*, 2000) and Huntington's disease (Ghavami *et al.*, 2014, Kim *et al.*, 2001, Mattson, 2000). Excess of apoptosis occurs in the cases of acute hypoxia and ischemia-reperfusion injury in cases of central nervous system insults (e.g., stroke) or acute and chronic heart diseases (Benchoua *et al.*, 2001, Whelan *et al.*, 2010) or chronic liver diseases (Fig. 2B) (Wang, 2014). Infectious agents have developed variety of mechanisms to interact with the host's defensive mechanisms in order to misuse them for spreading or evade the immune response. They may activate or inhibit apoptosis depending on the nature of the pathogen, cell type or pathogen abundance (Favaloro *et al.*, 2012). Many viruses code apoptosis inhibitors, such as human papillomavirus (HPV) (Fig. 2D) (Jiang and Yue, 2014, McNees and Gooding, 2002) or anti-apoptotic protein homologues (Pauleau *et al.*, 2007). Most of them cause excessive cell death by directly affecting RCD pathway proteins or indirectly, causing cell stress that may be detected by p53. In several autoimmune diseases, including pemphigus vulgaris (Deyhimi and Tavakoli, 2013), autoimmune lymphoproliferative syndrome (Madkaikar *et al.*, 2011), rheumatoid arthritis, systemic lupus erythematosus and Hashimoto's thyroiditis (Eguchi, 2001, Nagata, 2007), abnormalities of cell death routines are reported. Importantly, resistance to RCD is one of the major mechanisms of malignant transformation of cell. Disturbances of Bcl-2 family proteins are connected to B-cell lymphomas, Hodgkin lymphoma, breast cancer, lung cancer and kidney cancer (Egle *et al.*, 2004, Gascoyne *et al.*, 1997, Gobe *et al.*, 2002, Han *et al.*, 2002, Tawfik *et al.*, 2012). Apoptosome inactivation is frequently found in several cancer

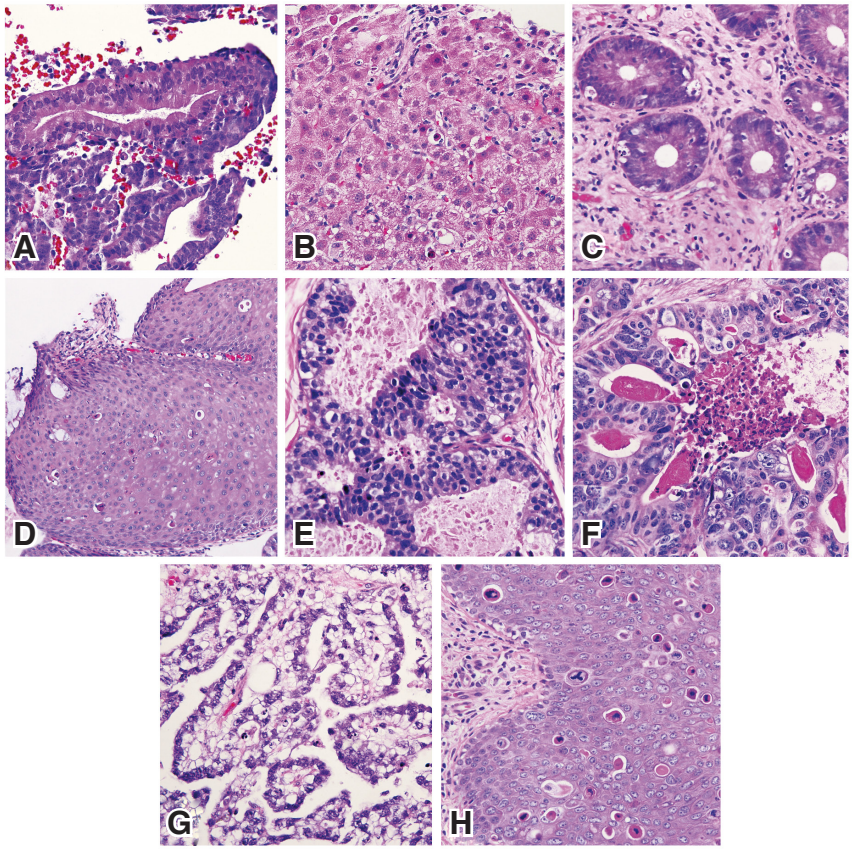

Fig. 2. Apoptosis and tissue turnover. *Apoptosis regulates normal tissue turnover in physiological endometrial proliferation* **(A)**, *but is observed in chronic liver disease* **(B)**, *Graft versus host disease* **(C)**, *HPV-related condylomas* **(D)**, *ductal carcinoma* in situ *of the breast* **(E)**, *colon cancer* **(F)**, *embryonal carcinoma of the testis* **(G)**, *and squamous cell carcinoma of the skin* **(H)**.

TABLE 2

TENTATIVE CELL DEATH MODALITIES OR PATHWAYS

Cell Death modality	Morphological and biochemical characteristics
Mitotic catastrophe (Vakifahmetoglu *et al*., 2008, Vitale *et al*., 2011)	Occurs during mitosis, induced by chromosomal or defects in mitosis machinery. It is an oncosuppresive mechanism that precedes cell death or senescence, rather than distinct cell death pathway. Affects single cells during mitosis. Characterized by micronucleation, multinucleation and apoptotic or necrotic like changes.
Cornification (Candi *et al*., 2005)	Cell death subroutine restricted to keratinocytes, necessary for the generation of stratum corneum of the epidermis, involving caspase-14.
Autosis (Liu and Levine, 2014)	Autophagy-dependent, non-apoptotic form of cell death induced by autophagy-inducing peptides, starvation, and hypoxia-ischemia. Characterized by enhanced cell substrate adhesion, focal ballooning of the perinuclear space, and dilation and fragmentation of endoplasmic reticulum.
Anoikis (Frisch and Francis, 1994)	Death of adherent cells initiated by lack of cell-to-matrix interactions, executed by intrinsic apoptotic pathway.
Entosis (Overholtzer *et al*., 2007)	Engulfment of a live cell by live neighboring cell. Engulfed cell dies through a lysosomal degradation within a phagosome.
Pyroptosis (Brennan and Cookson, 2000)	Caspase-dependant cell death pathway involving early activation of caspase-1. Shows both apoptotic and necrotic morphological features. Induced by several types of bacteria (e.g., *Salmonella typhimurium, Shigella flexneri, Listeria monocitogenes, Pseudomonas aeruginosa*, and others)
Parthanatos (Ame *et al*., 2004)	Caspase-independent cell death pathway. Induced by DNA damage. Involving the PARP family of enzymes (e.g., PARP1).
Netosis (Remijsen *et al*., 2011)	Cell death pathway restricted to granulocytes during their non-physiological stimulation and release of neutrophil extracellular traps (NETs, composed of nuclear chromatin, histones and granular antimicrobial proteins) which have antimicrobial activity. Shares the biochemical properties of autophagic cell death and regulated necrosis.

types including melanoma, leukemias, glioblastomas, cervical carcinoma and Burkitt's lymphoma (Favaloro *et al*., 2012). Death receptor defects are also reported in many cancer types, notably the melanoma, hepatocellular carcinoma, T-cell leukemias, endometrial, lung and colon cancer (Hahne *et al*., 1996, Johnstone *et al*., 2008, Shin *et al*., 2002). Altered caspase activity and inhibition of AIFs are also reported in many studies.

The complexity of cell death regulation and the large numbers of molecules involved, require several approaches for developing therapeutics to modulate the pathway. Potential strategies include, but are not limited to small molecules that inhibit or activate specific proteins involved in the pathway, antisense oligonucleotides directed against specific genes involved in cell death, fusion proteins that can activate or block cell membrane receptors to modulate the pathway, mitochondria specific agents, or modified nanoparticles that activate apoptosis (Cho, 2014, Huang *et al*., 2013, Murphy *et al*., 2003a, Murphy *et al*., 2003b). Eventually, many of the potential therapeutic agents reached phase I and II clinical trial (Wong, 2011).

Methods to detect regulated cell death

With the recent advances in biotechnology, a number of methods have become available for detection and quantification of specific cell death subroutines. Morphological methods have been dominating for years, due to their availability and simplicity. However, because of molecular differences between morphologically similar RCD subroutines, the NCCD recommended shift from morphological to molecular assays for detection and quantification of cell death (Galluzzi *et al*., 2012). Nevertheless, both morphological and molecular methods have their advantages and disadvantages, which are beyond the scope of this review. Broadly, methods for detecting the RCD are based on cytomorphological features, detection of caspases, cleaved substrates, regulators and inhibitors, DNA fragmentation, plasma membrane alterations and mitochondrial assays. Different technologies may be employed and they include transmission electron microscopy (TEM), standard light microscopy, immunohistochemistry and special stains, (immuno)fluorescent or luminescent assays, DNA electrophoresis, flow cytometry, western blot, or these technologies may be combined to achieve more specific results (Holdenrieder and Stieber, 2004, Otsuki *et al*., 2003). Recently the technology of live cell imaging has been gaining more attraction.

Cytomorphological detection of apoptosis, augmented by immunohistochemical markers, is commonly used in research and diagnostic histopathology. A set of useful immunohistochemical markers may be used to detect apoptotic cells in their early phase, when morphological changes are still unremarkable (Table 3). Those markers improved the major drawback of the morphological approach that was related to detection of apoptosis in relatively narrow time window from development of morphological signs to dissolution and removal of the cell. Some of the specific immunohistochemical markers of apoptosis will be discussed later in the context of diagnosis or prognosis.

Morphological approach was combined with detecting DNA fragmentation in TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling) assay (Ito and Otsuki, 1998). Labeled UTP is added to the 3′-end of the DNA fragments using terminal transferase. Subsequently, the dUTP can then be labeled with different probes to allow detection by light or fluorescence microscopy. TUNEL assay is not entirely specific for apoptosis, as it can detect DNA fragments in necrosis or cell undergoing DNA repair (Otsuki *et al*., 2003). Apoptosis may be detected by DNA isolation and agarose gel electrophoresis, which shows characteristic DNA fragments making DNA ladder. However, because the DNA fragmentation occurs in late stages, and it is not specific for particular RDC modality, it was depreciated by NCCD (Galluzzi *et al*., 2012).

Annexin V is a calcium-dependent protein that preferentially binds the phosphatidylserine, with high affinity. Conjugation of annexin V with a reporting system enables its detection on the membrane of the dying cell (van Heerde *et al*., 2000). However, phosphatidylserine translocation lacks the specificity and may occur in both apoptosis and necrosis. Combining this assay with nucleic acid dyes, which stain the DNA of cells with disrupted membrane, i.e. necrotic cells, may distinguish between two death modalities.

Mitochondrial assays measure mitochondrial permeability transition (MPT), depolarization of the inner mitochondrial membrane, Ca^{2+} fluxes, mitochondrial redox status, and reactive oxygen

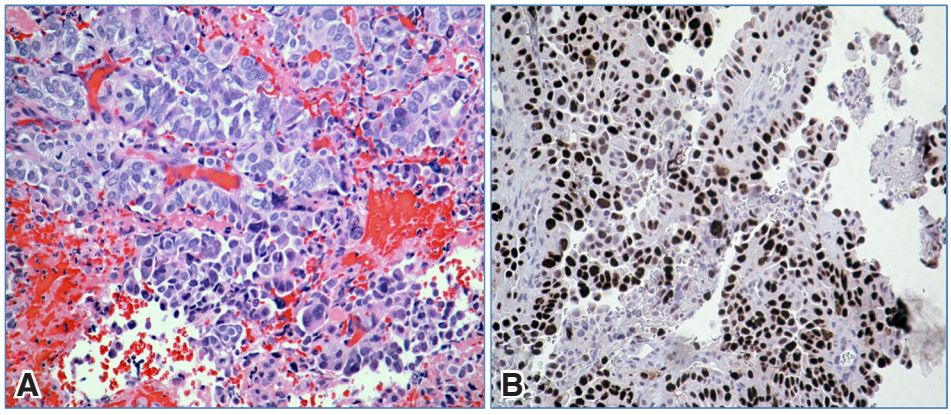

Fig. 3. High-grade serous adenocarcinoma of the endometrium (Hematoxylin and Eosin) (A) was diffuse and strong positive for p53 protein (B) (10x).

species. Also, the cytochrome *c* release or Bcl-2 family proteins at mitochondrial membrane may be detected by using confocal microscopy (Elmore, 2007).

Diagnostic implications

Although significant advances have been made in decrypting the programs of cell death, identifying the key players and potential therapeutic targets, the diagnostic, prognostic and predictive value of the biomolecules involved in RCD has been so far of limited extent.

The value of apoptotic index (AI) in diagnosis or prognosis has been studied in many cancer types (Becker *et al.*, 2014, Fu *et al.*, 2014, Gkogkou *et al.*, 2014, Kuriyama *et al.*, 2002, Ribeiro Mde *et al.*, 2004, Richter *et al.*, 2002, Wu *et al.*, 2013), but the results were controversial. Further, AI was investigated as a predictor of chemotherapy response in gastric carcinoma (Jia *et al.*, 2012, Wu *et al.*, 2014a) or radiotherapy response in cervical cancer (Bhosle *et al.*, 2005), and breast cancer (Burcombe *et al.*, 2005). Some of the studies found AI to be useful alone or in combination with other prognostic factors, while others failed to demonstrate that utility. Of non-neoplastic diseases, the estimation of apoptosis has found its diagnostic utility in the evaluation of graft versus host disease (GVHD). Acute phase of GVHD is defined as onset of severe abdominal symptoms including diarrhea, bleeding, vomiting, and abdominal pain with a histologic evidence of GVHD within 100 days of transplant or donor lymphocyte infusion (Ross and Couriel, 2005, Thompson *et al.*, 2006). Colon is typically involved, and biopsy of the distal part of colon shows apoptotic bodies that are required to make the diagnosis of GVHD (Ross, 2005) (Fig. 2C). Other parts of the gastrointestinal system can be involved as well. Skin and liver changes are also common features of this syndrome.

One of the most relevant diagnostic markers related to apoptosis is Bcl-2 protein. Bcl-2 has been found to be overexpressed in follicular center B-cell lymphoma due to the specific t(14;18) translocation. Bcl-2 overexpression may well be seen in a subset of other B-cell lymphoproliferative diseases including chronic lymphocytic leukemia, diffuse large B-cell lymphoma and mantle cell lymphoma as a consequence of hypo-methylation of the bcl-2 promoter or the loss of the specific miRNAs (Kelly and Strasser, 2011). In all cases, immunohistochemistry and in-situ hybridization assays may detect specific alterations of *BCL2*. Bcl-6 is

TABLE 3

BIOMARKERS OF RCD USED IN DIAGNOSTIC AND RESEARCH HISTOPATHOLOGY

Marker	Type	Use
Apoptotic index (AI)	Diagnostic	Diagnostic utility in GVHD (Ross, 2005). Prognostic utility controversial.
Bcl-2	Diagnostic, prognostic	Anti-apoptotic protein. Distinguishing follicular hyperplasia of lymph node from follicular lymphoma (Ozsan *et al.*, 2011, Weinberg *et al.*, 2009); Detecting immature enteric ganglion cells in pediatric intestinal pseudo-obstruction (Park *et al.*, 2005); Diffuse large cell lymphoma: possible adverse prognostic factor (Berglund *et al.*, 2005, Iqbal *et al.*, 2011); Myelodysplastic syndrome: increased expression associated with progression; Possible prognostic value in early stage breast cancer(Dawson *et al.*, 2010)
Bcl-6	Diagnostic, prognostic	Differential diagnosis of small B-cell lymphoma. Follicular lymphoma is bcl-6 (and CD10) positive whereas other small B-cell lymphomas are usually negative (Dogan *et al.*, 2000). In difficult cases of follicular lymphoma, bcl-6 can identify an interfollicular component. Important prognostic marker in DLBCL, where CD10, bcl-6 and MUM1/IRF4 are used to identify germinal center and activated B-cell phenotypes (Hans *et al.*, 2004). Valuable in distinguishing classical Hodgkin lymphoma from nodular lymphocyte predominant Hodgkin lymphoma (NLPHL). The Reed-Sternberg cells of classical Hodgkin lymphoma are bcl-6 negative whereas the large cells of NLPHL are bcl-6 positive (Kraus and Haley, 2000).
p53	Diagnostic, prognostic	Tumor suppressor gene. Used to differentiate malignant conditions, which often show p53 positivity (carcinoma in situ of urothelium and other sites, invasive carcinoma) from reactive and metaplastic conditions which are usually p53 negative (McKenney *et al.*, 2001). Useful to distinguish uterine serous carcinoma (p53+) from endometrioid carcinoma (usually p53-). Possible predictive value in thymic neoplasms (Khoury *et al.*, 2009). Unfavourable prognosis biomarker in esophageal SCC (Xu *et al.*, 2014). p53 expression in upper urinary tract urothelial carcinoma was correlated with advanced pathologic stage, high histologic grade and female gender (Lee *et al.*, 2014)
Fas	Prognostic	Reduced Fas expression is a poor prognostic factor for breast carcinoma, colorectal carcinoma, retinoblastoma and urothelial carcinoma (Botti *et al.*, 2004, Shanmugam *et al.*, 2003, Wang *et al.*, 2006, Yamana *et al.*, 2005). Reduced Fas expression is a favorable prognostic factor in primary nodal diffuse large B-cell lymphoma and subcutaneous panniculitis-like lymphoma (Eser *et al.*, 2006, Takeshita *et al.*, 2004)
FasL	Diagnostic	Positive in Reed-Sternberg cells of Hodgkin's lymphoma (nodular sclerosis and mixed cellularity (Verbeke *et al.*, 2001)
Active caspase-3	Apoptosis marker	Immunohistochemical marker of apoptosis (Gown and Willingham, 2002, Jeruc *et al.*, 2006, Sabine *et al.*, 2012)
Caspase-7	Apoptosis marker	Immunohistochemical marker of colonic carcinoma (Bressenot *et al.*, 2009, Palmerini *et al.*, 2001)
Caspase-8	Apoptosis marker	Marker of initiation of apopotosis (Bressenot *et al.*, 2009, Cho *et al.*, 2010, Palmerini *et al.*, 2001, Xu *et al.*, 2008)
Cleaved PARP	Apoptosis marker	Marker of apoptosis and paranathos. Cleaved by caspase-3. Poly (ADP-ribose) polymerase (PARP) is a nuclear enzyme involved in DNA repair (Donizy *et al.*, 2013, Sulzyc-Bielicka *et al.*, 2012, Wu *et al.*, 2014b)
Annexin V	Apoptosis marker	Marker of early phase of apoptosis. Binds to translocated phosphatydilseryne on the outer plasma membrane layer (Gerber *et al.*, 2000)

another important the Bcl family-related protein with a significant diagnostic and prognostic utility, particularly in hematopathology. Bcl-6 nuclear positivity indicates follicle cell origins in lymph node. Its over-expression is frequently observed in follicular lymphoma, diffuse large B-cell lymphoma (DLBCL) and Burkitt's lymphoma due to the underlying genetic alterations (e.g. translocations) that affect 3q27 locus (Chaganti *et al.*, 1998). Lymphomas belonging to the subset of DLBCL that lack 3q27 rearrangements frequently acquire mutations in *BCL6* promoter regions.

p53 protein has had a limited diagnostic utility although its overexpression correlates with the tumor differentiation (grade) and aggressiveness. In diagnostic pathology, it has been primarily used to discriminate between atypical (neoplastic) and reactive (non-neoplastic) proliferation (e.g. urothelial proliferation); to evaluate the extent of dysplastic changes (e.g. Barrett esophagus, dysplastic changes in inflammatory bowel disease, anal intraepithelial neoplasia, progression of squamous cell dysplasia of the respiratory epithelium (Demirovic *et al.*, 2014, Gattuso, 2009); to distinguish between the histotypes (serous that is typically p53+ vs. endometrioid carcinoma of endometrium/ovary, usually p53-) (Fig. 3) (Macwhinnie and Monaghan, 2004). A protein that inhibits p53 named Mdm2 (Murine Double-Minute 2) has also yielded diagnostic utility as it has been found to be specific for liposarcomas due to the *MDM2* gene amplification (detected by in-situ hybridization assays) with a good correlation with Mdm2 protein status determined by immunohistochemistry (Hav *et al.*, 2011). In addition, exposure of neoplastic cells to radiation- or chemotherapy agents may trigger apoptosis that is induced by DNA damage and activation of p53 protein (Kemp *et al.*, 2001).

As previously mentioned, apoptosis is a core mechanism of neuron cell death in various neurodegenerative diseases including Parkinson's disease, Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis as well as in stroke (Mattson, 2000), but diagnostic utility of apoptosis in these conditions is very limited.

Conclusions

A tremendous progress has been achieved in the last few decades in revealing the secrets of regulated cell death. Several molecular pathways and their interconnections build a complex cell signaling network with a numerous genes and proteins involved, to serve a function of proper tissue development and homeostasis. Dysregulation of these complex molecular mechanisms is involved in the pathogenesis of various disorders, including cancer. Therapeutical modulation of cell death routines may have wide implication on many of the diseases. Yet, the diagnostic utility of genes and proteins involved in regulated cell death is still of limited value in histopathology.

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