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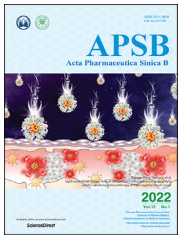

REVIEW

Preliminary evidence for the presence of multiple forms of cell death in diabetes cardiomyopathy

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Abstract Diabetic mellitus (DM) is a common degenerative chronic metabolic disease often accompanied by severe cardiovascular complications (DCCs) as major causes of death in diabetic patients with diabetic cardiomyopathy (DCM) as the most common DCC. The metabolic disturbance in DCM generates the conditions/substrates and inducers/triggers and activates the signaling molecules and death executioners leading to cardiomyocyte death which accelerates the development of DCM and the degeneration of DCM to heart failure. Various forms of programmed active cell death including apoptosis, pyroptosis, autophagic cell death, autosis, necroptosis, ferroptosis and entosis have been identified and characterized in many types of cardiac disease. Evidence has also been obtained for the presence of multiple forms of cell death in DCM. Most importantly, published animal experiments have demonstrated that suppression of cardiomyocyte death of any forms yields tremendous protective effects on DCM. Herein, we provide the most updated data on the subject of cell death in DCM, critical analysis of published results focusing on the pathophysiological roles of cell death, and pertinent perspectives of future studies.

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1. Introduction

The risk of heart disease increases 2−3 times in diabetic patients. Diabetic mellitus (DM) is a common degenerative chronic metabolic disease that causes considerable negative impacts on health and lifespan with diabetic cardiovascular complications (DCCs) as major causes of death in diabetic population[1−5]. Diabetic cardiomyopathy (DCM) is one of the DCCs which literally account for more than 80% of diabetic deaths. DCM is manifested as specific anomalies of myocardial structure and function in diabetic patients in the absence of overt clinical coronary artery disease, valvular disease, and other conventional cardiovascular risk factors, such as hypertension and dyslipidemia[1−5]. In 2013, the American College of Cardiology Foundation, the American Heart Association, and the European Society of Cardiology in collaboration with the European Association for the Study of Diabetes defined DCM as a clinical condition of ventricular dysfunction that occurs in the absence of coronary atherosclerosis and hypertension in DM patients[6].

DCM in the initial stages is generally without any symptoms. As the disease progresses, visible symptoms emerge, such as breathlessness even at rest, shortness of breath after physical activities, swelling in the ankles, feet, or abdomen, dizziness, light headedness, fatigue, irregular heartbeat, angina, etc. DCM is associated with both type 1 and type 2 DM (T1DM and T2DM, respectively)[7]. Although DCM is common, its clinical diagnosis is rather difficult because overt DCM takes years to develop. Diagnosis of DCM involves detections of both structural and functional alterations in the left ventricle (LV) and exclusion of other heart disease or comorbidities as a potential etiology for myocardial dysfunction. The most frequently used diagnostic methods are echocardiography and cardiac magnetic resonance imaging (MRI) to identify the cardiac anomalies at the early stages before any heart failure (HF) symptoms emerge[8,9]. Evidence of cardiac hypertrophy can be detected by diastolic dysfunction by transmitral Doppler or tissue Doppler, which is essential for the diagnosis of DCM. In addition, myocardial performance index (MPI) for evaluating global cardiac contractility is also considered of diagnostic value for DCM, since an altered MPI is the earliest echocardiographic change in DCM with recorded higher values in DCM patients compared with controls[10].

As an independent risk factor of HF, DCM is generally characterized in the early stage by myocardial fibrosis, left ventricular (LV) hypertrophy, adverse remodeling, and advancing diastolic dysfunction. In the advanced stages DCM degenerates into systolic dysfunction with reduced ejection fraction (EF) and eventually by clinical HF[11]. In the early phase, the pathologic changes of DCM can be reversible with strict metabolic control, but in the continuous process the myocardial changes become irreversible with increased risk of developing HF[12,13]. There has not been any effective specific treatment available for DCM to date, though new approaches have been examined in animal models[14]. At present, clinical treatment centers around intense glycemic control through diet, oral hypoglycemics and frequently insulin and management of HF symptoms. In addition, other pharmacological therapies have also been used, such as RAAS blockers, β-blockers, statins, antioxidants, advanced glycation end-product inhibitors (aminoguanidine, pyridoxamine), and advanced glycation end-product cross-link breakers (alanine, aminotransferase 711)[8,9].

The development and progression of DCM has been associated with a complex plethora of regulatory factors and signaling pathways. These factors include hyperglycemia, insulin resistance, mitochondrial dysfunction, endothelial dysfunction, excessive oxidative stress/accumulation of reactive oxygen species (ROS), endoplasmic reticulum stress, elevations in advanced glycation end products (AGEs), inflammation, extracellular matrix (ECM) deposition, fibrosis, impaired intracellular calcium handling, renin−angiotensin−aldosterone system (RAAS) activation, cardiac autonomic neuropathy, microvascular dysfunction, and a myriad of cardiac metabolic abnormalities[1−5]. The prevalence of HF in diabetic patients ranges from 19% to 26%[11,15−17]. All these detrimental alterations can cause cell death, contributing to decreased cardiac muscle mass, interstitial fibrosis, impaired cardiac function and the degenerative nature of HF[18]. Indeed, increased cardiac cell death has been regarded as a major risk factor for the development of DCM; biopsied diabetic heart tissue expresses 85-fold more cardiomyocyte apoptosis than control non-diabetic hearts[19]. This article aims to advocate and stimulate the research activities pertinent to cell death in DCM by presenting an overview on various forms of cell death occurring in DCM by shedding light on the current status of the related studies, discussing the role of cell death in the development of DCM, stressing the unanswered questions in regards with the topic, and providing critical evaluation of the existing data and future direction of the research field.

2. Diverse forms of cell death

One of the mechanisms by which multicellular organisms maintain their cell homeostasis is cell death, which is an ancient process with fundamental biological importance in both normal physiology and pathological states with physiological benefits and pathological consequences. While programmed cell death was once thought to occur only by apoptosis (type I cell death)[19−22], an array of alternative forms has been identified that, like apoptosis, are delicately controlled by genetic machinery and contribute to cell turnover in normal or pathophysiologic contexts. These new types of cell death include autophagic cell death (type II cell death; *e.g.*, autosis)[23−25] and programmed forms of necrosis (type III cell death; *e.g.*, necroptosis)[26−29], entosis (type IV cell death)[30−33], ferroptosis[34−37], etc. The characteristics of several forms of cell death which have been identified in DCM are summarized in Table 1 for comparisons.

The Nomenclature Committee of Cell Death (NCCD) recommended in 2012 that researchers replace the morphologic classification of cell death with a new classification system based on molecular events associated with cell death[26]. Programmed or elective cell death is required for proper tissue development and homeostasis by eliminating unnecessary, aged, or damaged cells[38], as well as for counteracting pathological processes such as carcinogenesis[39], viral infection[40] or myocardial ischemia[41,42] by killing transformed or damaged cells. Cell death is tightly regulated, which is defined as a death process that relies on dedicated molecular machinery. The molecular machinery is modulated by a variety of regulatory factors in the signaling network and can be artificially manipulated by specific pharmacological and genetic interventions. Programmed cell death is mechanistically distinct from necrosis, or other unregulated cell death caused by environmental stresses.

The maintenance of the normal structure and function of the cardiovascular system requires a balance between cell formation and death in the vascular and cardiac tissues which are composed of endothelial cells, vascular smooth muscle cells, cardiomyocytes, and cardiac fibroblasts. Excessive cell death often

leads to structural destruction and functional impairment of the cardiovascular system.

2.1. *Apoptosis*

Apoptosis, also called programmed cell death (PCD), is the most thoroughly characterized and the fastest and irreversible form of cell death which is biochemically characterized and mechanistically defined by the activation of particular cysteine-dependent aspartate-specific proteases (caspases)-induced proteolytic cascade[43–46]. It is featured with cytoplasmic shrinkage, nuclear pyknosis, karyorrhexis, DNA fragmentation, and plasma membrane blebbing (Table 1). The contents of apoptotic cells are packaged into membrane-enclosed apoptotic bodies, which are targeted for phagocytosis and removal *in vivo* without involvement of inflammatory response. Apoptosis is the most widely recognized program of cell death, which produce an orchestrated disassembly of the cell. In general, two main apoptotic signaling pathways are operating in organisms: the extrinsic apoptotic pathway and the intrinsic apoptotic pathway (Fig. 1).

The extrinsic apoptotic pathway is also known as the death receptor pathway, which is characterized by binding of multiple death ligands, primarily tumor necrosis factor-α (TNF-α) and FAS, to their homologous receptors. The binding forms the multiprotein death-inducing signaling complex (DISC) *via* the intermediate membrane proteins TNF receptor-associated death domain (TRADD) and FAS-associated death domain protein (FADD), respectively, and triggers apoptotic signal[47–51]. This is followed by the activation of caspases-8 and -9 which subsequently activate the apoptotic executioner caspases-3, -6, and -7, ultimately leading to apoptosis.

The intrinsic pathway is known as mitochondrial death pathway[52–57]. This pathway is characterized by the formation of a megapore across both the inner and outer membranes of the mitochondria by a group of proteins called the complex permeability transition pore (CPTP). This step depolarizes mitochondrial membrane and makes it leaky allowing mitochondrial proteins such as cytochrome *c* to release from the intermitochondria membrane space into the cytosol. Released cytochrome *c* combines with apoptosis protease activating factor-1 (APAF-1) to form a complex named "apoptosome" which serves as a platform for the cleavage and activation of downstream caspase-9[57].

In general, regenerative diseases are associated with abnormally decreased apoptosis, whereas degenerative pathological processes are related to abnormally increased apoptosis[43–46]. For example, over 50% of neoplasms have defects in apoptotic machinery, and HF is characterized by tremendous apoptosis[58–61].

2.2. *Autophagy and autosis*

Autophagy is characterized by extensive cytoplasmic vacuolization (single- or double-membrane lysosomal-derived vesicles) to form autophagosome, phagocytosis and subsequent lysosomal degradation (Table 1)[62]. Under physiological conditions, it is a process by which cells recycle their own non-essential, redundant, or damaged organelles and macro-molecular components[63–65]. However, autophagy is also involved in tumor suppression, deletion of toxic misfolded proteins, elimination of intracellular microorganisms, and antigen presentation. Autophagosomes are the hallmark of autophagy, and their formation is initiated by phosphatidylinositol-3-kinase (PI3K) and autophagy-related proteins (ATG)-6 (also known as Beclin-1) and regulated by a complex set of evolutionarily conserved ATG[63,65]. Once formed, the autophagosomes fuse with lysosomes. On the other hand, autophagosome formation is negatively regulated by the mammalian target of rapamycin (mTOR), a serine/threonine protein kinase. Physiological levels of autophagy promote cellular survival in response to a variety of stress conditions, including starvation, hypoxia, mitochondrial damage, and pathogen infection[63,64,66]. Excessive or unbridled autophagy, however, has been linked to various forms of caspase-independent cell death coined "autophagic cell death"[65]. Nevertheless, the evidence linking autophagy to cell death in these early reports is largely circumstantial, and the observations reported to date have been controversial. Especially, genetic deletion of key autophagic genes accelerates rather than inhibits cell death, emphasizing the predominant survival role of autophagy[65,67]. These results are in apparent contradiction to the most recent guidelines from the Nomenclature Committee on Cell Death, which specifies that autophagic cell death should be identified only if the process is blocked by genetic interventions targeting at least two components of the molecular machinery of autophagy[26]. While *in vivo* evidence is still lacking, autophagic cell death has been identified in higher eukaryotes under *in vitro* conditions[68–74]. More often, therefore, autophagic cell death refers to cells that exhibit massive numbers of cytoplasmic autophagic vacuoles but lack signs of apoptosis. Such a definition does not distinguish whether these autophagosomes facilitate cell death or represent a cell that can no longer compensate by sacrificing vital components, with the latter referred to as "autophagy-associated cell death" rather than "autophagy-induced" cell death. Importantly, autophagic cell death is only revealed in the absence of apoptotic pathways.

Autosis ("auto" represents autophagic, and "tosis" means death) is an autophagy-dependent non-apoptotic form of cell death. It is characterized by enhanced cell substrate adhesion, focal ballooning of the perinuclear space, dilation, and fragmentation of endoplasmic reticulum (ER), and nuclear membrane convolution in the early phase and focal swelling of the perinuclear space in a later stage. Autosis can be triggered by autophagy-inducing peptides, starvation, and neonatal cerebral hypoxia-ischemia, and suppressed by pharmacological inhibition of Na^+/K^+-ATPase (*e.g.*, cardiac glycosides) or genetic inactivation of the gene encoding Na^+/K^+-ATPase *in vitro* and *in vivo*[23–35,75–77].

According to Liu et al.[23], autotic death can be identified by several criteria: (1) the absence of morphological, biochemical, and genetic evidence for other cell death pathways, (2) unique morphological changes, and (3) a unique dependence on Na^+/K^+-ATPase. While autosis is accompanied by several stereotypic morphological features, the "sine qua non" of autosis is the nuclear membrane changes. Although like in apoptosis, chromatin condensation also occurs in autosis, it is very mild with neither DNA laddering nor TUNEL-positive staining[23]. Another unique feature of autosis compared with other forms of cell death is the increased substrate adherence of dying cells. Most importantly, autosis, but not apoptosis or necrosis, is suppressed by pharmacological or genetic inhibition of Na^+/K^+-ATPase. Conversely, treatment with caspase or RIP kinase inhibitors or genetic deletion of pro-apoptotic BAX and BAK or pro-necroptotic RIPK1 and RIPK3 does not protect cells against autotic cell death. Unlike necrosis or necroptosis, ROS is not a mediator in autosis[23]. Thus,

Table 1 Comparisons among various types of cell death.

Cell death	Apoptosis	Pyroptosis	Autophagic cell death	Autosis	Necroptosis	Ferroptosis
Definition	A form of caspase-mediated programmed cell death	A highly inflammatory form of programmed cell death	A form of non-programmed, autophagosome-dependent cell death lack signs of apoptosis	An autophagy- and Na^+/K^+-ATPase-dependent non-apoptotic form of cell death	A programmed form of necrosis, or inflammatory cell death	An iron- and lipotoxicity-dependent form of regulated cell death mainly caused by oxidative stress
Pathophysiologic relevance	In general, apoptosis is decreased with regenerative diseases, but increased in degenerative pathological processes	Protects against infection and induces pathological inflammation	May underly the pathological conditions of cancer and autoimmune diseases by altering metabolic conditions	Involved in cerebral and cardiac hypoxia-ischemia and reperfusion injuries	A viral defense mechanism, promoting "cell suicide" in the presence of viral caspase inhibitors to restrict virus replication	Neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease associated with excessively increased iron and ROS in brain
Cellular role	Remove damaged cells	Crucial for controlling microbial infections	Recycle non-essential, redundant, or damaged organelles and macro-molecular components	Abnormal cell death in ischemia and/or hypoxia	A viral defense mechanism for restricting virus replication	Regulatory role in growth of tumor cells and death of brain cells in the human body
Formation of complex	Formation of APAF-1 apoptosome, a multimeric protein complex combined with cytochrome *c* and bound to caspase-9 in the mitochondrial death pathway	Formation of NLRP3 inflammasome → caspase-1; Lipopolysaccharide → caspase-11	Formation of autophagosome with extensive cytoplasmic vacuolization, followed by phagocytosis and lysosomal degradation	Formation of autophagosome with enhanced cell substrate adhesion and swelling of the perinuclear space	Activation of RIPK1 and RIPK3 and formation of necrosome with active disintegration of mitochondrial, lysosomal and plasma membranes	ACSL4 upregulation; ROS formation by ferroptosis activator erastin
Caspase activation	Activation of caspases-8 or 9 and subsequent activation of caspases-3/6/7	Activation of caspase-1/4/5	Without caspase activation	Without caspase activation	Caspase inactivation-dependent; Formation of caspase-8−FLIP heterodimers; ROS as a mediator	Without caspase activation
Plasma membrane	act membrane with blebbing; formation of apoptotic bodies	Rapid rupture of plasma membrane and release of proinflammatory molecules	Rupture and rare blebbing	Focal rupture	Rupture of cell membrane, translucent cytoplasm, swelling of organelles, and release of cell contents	Iron-dependent peroxidation of polyunsaturated phospholipids on cell membranes; opening of plasma membrane pore
Nucleus	Nuclear compaction and fragmentation	Intact nucleus	Minor changes without extensive condensation of the nucleus	Nuclear membrane convolution and shrinkage; focal concavity of the nuclear surface; focal ballooning of perinuclear space	Minor changes	Minor changes
Chromatin	Marked chromatin condensation	Marked chromatin condensation	Partial chromatin condensation	Mild chromatin condensation	Intact chromatin structure	No concentration of chromatin

Mitochondria	Minor changes with intact mitochondria; mitochondrial death pathway	Intact mitochondria	Occasional enlargement of mitochondria	Condensation with abnormal structure followed by subsequent swelling	Minor changes with intact mitochondria	Reduction or disappearance of mitochondrial cristae
Endoplasmic reticulum	Minor changes in morphology; ER stress as an inducer	Minor changes in morphology; ER stress as a contributor	Occasional enlargement of ER and ER stress as an inducer	Early dilation and fragmentation followed by ER disappearance	ER stress as an inducer	Minor changes in morphology; ER stress as a contributor
Inhibition	Caspase-3/9 inhibitors and inhibition of any other mediators along the death pathways	Caspase-1/4/5 inhibitors and inhibition of inflammasome and proinflammatory molecules	Inhibition of autophagy or the formation of autophagosome	Pharmacological inhibition by blockers or genetic inactivation of Na^+/K^+-ATPase; inhibitors of autophagy	RIPK1 and RIPK3 inhibitors	Ferroptosis inhibitors, such as ferrostatin-1, liproxstatin-1 and vitamin E, and iron chelators
Other features	Rounding up of cells; detachment from substrate; formation of apoptotic bodies	NLRP3 cleaves gasdermin D to generate an N-terminal gasdermin D fragment and activates caspase-1	Occasional enlargement of Golgi; Depletion of organelles	Enhanced cell-substrate adhesion	Rupture of cell membrane, translucent cytoplasm, swelling of organelles, and release of cell contents	Golgi stress

autosis utilizes an exclusive death machinery to initiate or execute cell death that is distinct from other forms of cell death. However, no biochemical markers are currently available to identify cells dying by autosis.

2.3. *Pyroptosis*

Pyroptosis, or caspase 1-dependent pro-inflammatory programmed cell death, is triggered by various pathological stimuli, such as stroke, heart attack or cancer, and is crucial for controlling microbial infections[78–83]. The requirement of inflammatory caspases in executing pyroptosis distinguishes it from another necrotic and inflammatory form of programmed cell death called necroptosis, which is independent of caspases. Pyroptosis is characterized by the formation of membrane pores, cell lysis and the release of pro-inflammatory cytokines and intracellular content, and by the activation of inflammasome, a molecular platform resulting in caspase-1 activation and interleukin (IL)-1β and IL-18 secretion upon cellular infection or stress[84]. Caspase-1 was first recognized as a protease that processes the inactive precursors of IL-1β and IL-18 into mature inflammatory cytokines[85]. However, caspase-1 activation can result not only in the production of activated inflammatory cytokines, but also rapid cell death. Caspase-1 activation is induced by the activation of nucleotide binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome, the key component of pyroptosis. The activated caspase-1 cleaves gasdermin D (GSDMD) to generate an N-terminal GSDMD fragment leading to the formation of membrane pores and subsequent inflammatory responses. An inflammasome is a macromolecular protein complex composed of inflammasome-initiating sensors (nod-like receptor proteins NLRP1, NLRP3, NLRC4, AIM2 or pyrin) and inflammatory caspases.

Pyroptosis possesses several morphological features of apoptosis; however, the mechanism, characteristics and outcome of caspase 1-dependent cell death are distinct from apoptosis (Table 1)[82–84]. Thus, the term pyroptosis (from the Greek "pyro", relating to fire or fever, and 'ptosis', meaning a falling) is used to describe the inherently inflammatory process of caspase-1-dependent programmed cell death[80]. As an innate immune effector mechanism, pyroptosis has the ability to defense against infection. It is a highly regulated cell death process, and inhibition of this process by pharmacological or genetic intervention is cardioprotective under many conditions. Emerging evidence has indicated that pyroptosis and related inflammasome activation play important roles in the progression of vascular inflammation and cardiovascular diseases[78,79,85].

2.4. *Necroptosis*

Various stimuli can induce a new form of non-apoptotic cell death called necroptosis, which resembles necrosis morphologically, but differs substantially in that it is a programmed regulated active type of cell death (Table 1)[29,86–90]. In addition, necroptosis also shares some features with apoptosis, or the two are deeply intertwined: many of the stimuli that trigger apoptosis, under conditions of caspase inhibition, can induce necroptosis. The recent identification of key molecules clarifies that necroptosis is distinguishable from the other mechanisms because it involves active cell death triggered by specific signaling pathways rather than non-specific injury[91]. Necroptosis

requires the protein kinase receptor-interacting serine/threonine-protein kinases 1 and 3 (RIPK1 and RIPK3, respectively)[68,91,92]. The active disintegration of mitochondrial, lysosomal and plasma membranes is involved in the process of necroptosis[91–93].

The best-characterized inducers of necroptosis are death receptor ligands, in particular TNF-α and FAS[94]. Binding of TNF-α to TNF receptor 1 (TNFR1) can result in three divergent functions: cell survival, apoptosis, or necroptosis by forming three distinct signaling complexes, respectively. In general, complex I is pro-survival, complex IIa pro-apoptotic, and complex IIb pro-necroptotic[94]. Receptor-interacting protein 3 (RIP3) is regarded as a critical regulator of necroptosis and RIPK3 is essential for TNF-induced necroptosis[95]. During the initiation of necroptosis, TNF receptor–ligand binding leads to an interaction between RIPK1 and RIPK3[96], leading to the activation of RIPK1 and RIPK3 and the subsequent formation of a complex called the necrosome.

Necroptosis is involved in the pathogenesis of many diseases, including neurodegeneration[97], cancer[98], and viral infection[99]. Moreover, necroptosis also exerts an important effect on cardiovascular disease[29,89,100]. Suppression of necroptosis can reduce arterial plaque formation, alleviate ischemia-reperfusion injury, and improve ventricular remodeling[101,102].

2.5. *Ferroptosis*

Ferroptosis is defined as an iron-dependent form of regulated cell death, which occurs through the lethal accumulation of lipid-based ROS when glutathione (GSH)-dependent lipid peroxide repair systems are compromised[34,37,103]. Ferroptosis involves genetic, metabolic, and protein regulators, triggers, and execution mechanisms that for the most part do not overlap with other forms of regulated cell death[103]. Ferroptotic cell death can be inhibited by lipophilic antioxidants, iron chelators, inhibitors of lipid peroxidation, and depletion of polyunsaturated fatty acyl phospholipids (PUFA-PLs), which are prime substrates driving lethal lipid peroxidation[37,104–106]. Clearly, ferroptosis is tightly related to amino acid, glutathione, lipid, and iron metabolisms.

The term ferroptosis was coined in 2012[103] to describe the form of cell death induced by the small molecule erastin, which inhibits the import of cystine, leading to glutathione depletion and inactivation of the phospholipid peroxidase glutathione peroxidase 4 (GPX4)[107]. Ferroptotic agents induce the unfolded protein response and subsequent ER stress-mediated activation of the PERK–eIF2α–ATF4–CHOP pathway. The CHOP (C/EBP homologous protein) signaling pathway-mediated P53-independent PUMA (P53 upregulated modulator of apoptosis) expression is involved in the synergistic interaction between ferroptosis and apoptosis[108].

A normal physiological function for ferroptosis as an adaptive and programmed form of cell death has not been established. However, ferroptosis has several connections to pathological cell death. A complex set of processes, described below, can drive or suppress lethal lipid peroxidation. Studies have suggested that ferroptosis is activated by degenerative processes or induced by anti-cancer therapy[103]. Inhibitors of ferroptosis, such as ferrostatins and liproxstatins, protect from ischemic injuries in the liver[109], kidney[110,111], brain[112], stroke[112], and heart (cardiomyopathy, HF)[113–115].

Evidence, either supportive or conclusive, has been emerging for the presence of various forms of cell death in DCM. The major signaling pathways leading to these DCM-associated forms of cell death are illustrated in Fig. 1.

3. Apoptosis in diabetic cardiomyopathy

The role of cardiomyocyte apoptosis in the development of DCM and other diabetes-associated cardiac conditions (such as myocardial infarction, HF, etc.) has been well established. Increased cardiac apoptosis has been considered a major risk

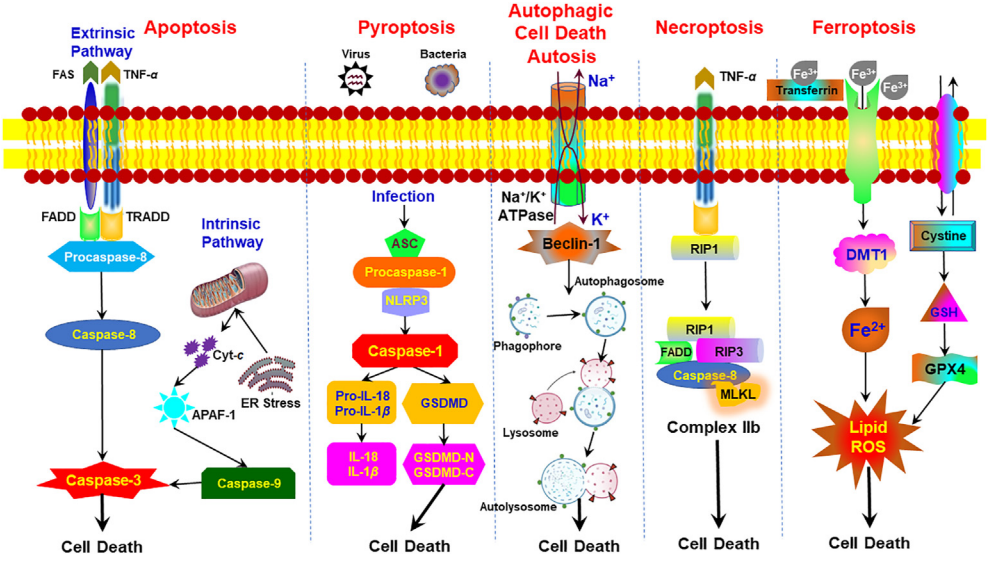

Figure 1 Schematic illustration of the major signaling pathways leading to various forms of cell death, which has been identified to date in diabetic cardiomyopathy (DCM), for straightforward comparisons. APAF-1, apoptosis protease activating factor-1; ASC, PYCARD protein; Beclin-1, autophagy-related proteins (ATG)-6; Cyt-*c*, cytochrome *c*; DMT1, divalent metal transporter 1; ER, endoplasmic reticulum; FADD, FAS-associated death domain protein; GSDMD, gasdermin D; GSDMD-N, N-terminal domain of gasdermin D; GSDMD-C, C-terminal domain of gasdermin D; GSH, glutathione; GPX4, the phospholipid peroxidase glutathione peroxidase 4; IL, interleukin; MLKL, mixed lineage kinase domain like pseudokinase; NLRP3, Nod-like receptor (NLR) family pyrin domain containing 3; ROS, reactive oxygen species; RIP1/3, receptor-interacting protein 1/3; TRADD, TNF receptor-associated death domain (TRADD).

factor for the development of DCM in both T1DM and T2DM models and T2DM patients, as supported by the evidence that biopsied heart tissue of patients with DCM expresses 85-fold more cardiomyocyte apoptosis than control nondiabetic hearts[19,116]. Cardiomyocyte apoptosis occurs in relatively late stage of DCM after long-term hyperglycemia in diabetic patients, leading to decrease cardiac contractile function and to ultimately promote cardiac remodeling due to cell loss[5,116]. However, *in vitro* studies demonstrated that apoptosis could be induced by short-term exposure to high glucose (HG)[117,118]. Both of the extrinsic TNF-α apoptotic pathway and intrinsic mitochondria-dependent death pathway are involved in apoptosis in DCM[119,120].

Hundreds of studies have documented that diabetes can disrupt the anti-apoptotic intracellular signaling cascades involved in myocardial protection and, the cell loss due to apoptosis decreases cardiac contractile function and promotes cardiac remodeling, ultimately leading to HF[33,116,120–126]. DCM is a metabolic disease that produces diverse apoptotic inducers such as hyperglycemia, mitochondrial damage and dysfunction, energy metabolic disturbance, excessive ROS, ER stress, AGEs, inflammation, impaired intracellular calcium handling, RAAS activation, etc. These diabetes-induced metabolic disturbances render cardiomyocytes under strong proapoptotic stress to commit cell death through multiple signaling pathways including both extrinsic and intrinsic pathways, as well as other related signaling cascades. In addition, apoptosis in DCM is modulated by numerous diverse cellular factors (ion channel protein, chemokine receptor, enzyme, hormone, RNA-binding protein, tumor suppressor, oncogene) and therapeutic agents.

In rats with DM induced by streptozotocin (STZ; T1DM), the mRNA levels of PI3K and Akt were found significantly decreased with concomitant upregulation of glycogen synthase kinase-3β (GSK-3β) expression, elevation of the ratio of cleaved-caspase 3/caspase-3 and BAX/BCL-2, and apoptotic cardiac cell death. Inhibition of expression of GSK-3β by lithium chloride (LiCl) attenuated the myocardial injury and apoptosis induced by DM[21].

The mitochondrial calcium uniporter (MICU1), a mitochondrial inner membrane Ca^{2+} channel and a key regulator of mitochondrial Ca^{2+} uptake, is essential in preventing mitochondrial Ca^{2+} overload. MICU1 is also known to cause excessive production of ROS, thereby playing important roles in regulating mitochondrial oxidative phosphorylation and redox balance. A study demonstrates that MICU1 is downregulated in *db/db* (T2DM) mouse hearts, which contributes to myocardial apoptosis in diabetes, whereas the reconstitution of MICU1 inhibits the development of DCM and improves cardiac function[127]. These findings suggest MICU1 an anti-apoptotic ion channel protein that might act to counter the mitochondrial death pathway.

The mammalian sterile 20-like kinase 1 (MST1) is a cytoplasmic kinase that acts upstream of the stress-induced mitogen-activated protein kinase (MAPK) cascade. A study reported that *Mst1* overexpression promotes cardiomyocyte apoptosis and impairs cardiac function *via* enhancing Beclin1 binding to BCL-2 to induce dissociation of BCL-2 from BAX in transgenic mice, whilst *Mst1* knockout produces the opposite effects, indicating MST1 a pro-apoptotic enzyme in DCM[128]. The same research group later showed that melatonin, a hormone that helps maintain circadian rhythm, reduces apoptosis and alleviates mitochondrial dysfunction by inhibiting MST1 phosphorylation and promoting SIRT3 expression in DCM[129]. Another group found that LIN28A, a LIN-28 family RNA-binding protein that acts as a post-transcriptional regulator of genes involved in developmental timing and self-renewal in embryonic stem cells, protects against diabetic cardiomyopathy through MST1 inhibition[130]. Their study further clarified that LIN28a increases the expression of AKT and inhibits the activation of MST1-mediated apoptotic pathways.

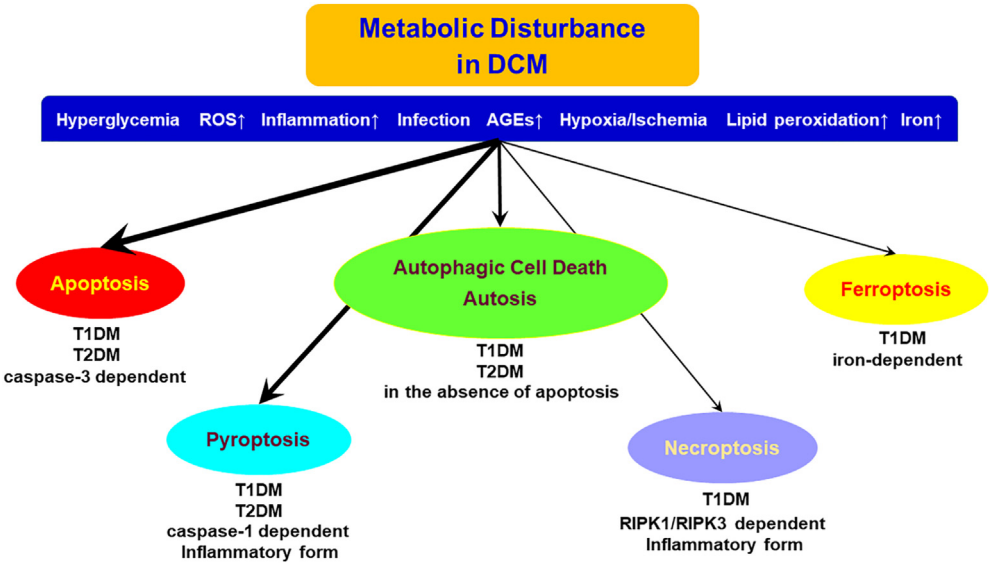

Figure 2 Schematic diagram depicting the relative importance of various forms of cell death identified by far in DCM induced by T1DM or T1DM and T2DM. The thickness of arrows represents the relative importance of different types of cell death in DCM, as indicated by the currently available data in the literature. The diagram also presents the most important inducers of cell death generated by metabolic disturbance in diabetic heart, with each of the inducers being able to induce more than one form of cell death. T1DM and T2DM: type 1 and type 2 diabetes mellitus, respectively.

In addition to inhibiting MST1 phosphorylation, melatonin can also ameliorate cardiac ER stress and the associated cardiomyocyte apoptosis in DCM in rats with T2DM[131]. The molecular and signaling mechanisms appear to be related to the repression of the expression of ER stress hallmarks, including CCAAT/enhancer-binding protein homologous protein, glucose-regulated protein 78, protein kinase RNA-like endoplasmic reticulum kinase (PERK). In addition, activation of transcription factor 6α in cardiac tissues, as well as reversal of downregulated BCL-2 and upregulated caspase-3 by melatonin also suppresses cardiomyocyte apoptosis in DCM.

The study by Gu et al.[132] examined their hypothesis that the development of DCM is attributable to P53-mediated early cardiac cell death and persistent cell senescence in a mouse model of T1DM. Their data unraveled that inhibition of P53 by its specific inhibitor pifithrin-α (PFT-α) effectively prevents the occurrence of early-stage apoptosis and subsequent cell senescence[132].

LAZ3 was initially identified as an oncogene in B-cell lymphomas and was later found to be a powerful transcriptional repressor that silences hundreds of genes. A study carried out in a mouse model of STZ-induced DM and a cellular model of hyperglycemia demonstrated that LAZ3 is downregulated in diabetic myocardium and cultured cardiomyocytes[133]. Artificial knockdown of LAZ3 by siRNA induces oxidative stress and cardiomyocyte apoptosis, whereas overexpression of LAZ3 by infection with adeno-associated virus (AAV9)-LAZ3 protects against apoptosis. LAZ3 enhances the activation of PPARα and PGC-1α activation, as well as subsequent NRF2 upregulation and nuclear translocation. Furthermore, it appears that LAZ3 promotes PPARα activation by decreasing miR-21 expression[133]. The results support the LAZ3/miR-21/PPARα/NRF2 signaling axis in the regulation of cardiomyocyte apoptosis in the context of DCM.

C−C chemokine receptor type 2 (CCR2), a receptor for monocyte chemoattractant protein-1, a chemokine which specifically mediates monocyte chemotaxis involved in inflammatory diseases, has been proven to play an important role in diabetes[135]. The expression of CCR2 was found significantly upregulated in the hearts of STZ-induced diabetic mice. Deletion of CCR2 inhibits cardiomyocyte apoptosis and improves cardiac dysfunction in *db*/*db* mice[134].

Excessive activation of the renin-angiotensin system (RAS) in DCM provokes a series of structural and functional abnormalities and causes ventricular remodeling and HF in diabetes (Pro)renin receptor (PRR) is a component of the RAS and has been reported to be upregulated in some cardiovascular diseases. It was documented that PRR overexpression promotes cardiomyocyte apoptosis and exacerbates myocardial in rats with DCM; conversely, PRR knockdown alleviates apoptosis and reverses the impaired cardiac function[135].

Hydrogen sulfide (H_2S), an endogenous antioxidant gaseous signaling molecule, can interact with proteins present in body tissue and the circulation[136]. Plasma H_2S levels are reportedly decreased in DM patients and mice[137], and preclinical studies revealed that exogenous H_2S treatment ameliorates DCM[138]. One of the beneficial actions of exogenous H_2S was found to be attributable to its anti-apoptotic property in diabetic hearts[139,140]. This cardioprotective effect of H_2S was also verified in H9C2 cardiomyoblasts and neonatal rat cardiomyocytes treated with the slow-releasing H_2S donor GYY4137 prior to exposure to HG and in diabetic mice as well. Echocardiographic and histopathological data demonstrated that exogenous H_2S improves cardiac function. Moreover, H_2S mitigates HG-induced oxidative stress and apoptosis in cardiac tissue and neonatal rat cardiomyocytes[141]. Furthermore, H_2S induces FOXO1 phosphorylation and nuclear exclusion *in vitro* and *in vivo*, which might mediate the beneficial effects of H_2S[142].

Apart from proteins, non-coding RNAs (ncRNAs) such as microRNAs (miRNAs or miR) and long non-coding RNAs (lncRNAs or lncR) also actively participate in the regulation of apoptotic cell death in the setting of CDM. For example, as described above miR-21 mediates the cardioprotective effect of LAZ3 against cardiomyocyte apoptosis[134].

Li et al.[142] found that forced expression of lncRNA-H19 inhibits miR-675 by the competitive endogenous RNA (ceRNA) mechanism to downregulate its target gene voltage-dependent anion channel 1 (VDAC1), rescuing cardiomyocyte apoptosis through suppressing the mitochondria-mediated apoptotic pathway. Another study exhibited that miR-186-5p overexpression suppressed apoptosis in HG-treated cardiomyocytes[118].

Xing et al.[143] reported that miR-207 is significantly upregulated in the myocardium of DCM mice. In cultured cardiomyocytes, miR-207 suppresses autophagy by increasing P62 and LC3II expression and promotes apoptosis by increasing caspase-3 activation. In diabetic cardiomyopathy of T2DM mice, miR-207 similarly inhibits autophagy and promotes apoptosis of cardiomyocytes by directly targeting lysosomal-associated membrane protein 2 (LAMP2), an autophagy-related protein[143].

The expression of lncRNA-*TINCR* in myocardial biopsies and serum samples is significantly lower in patients with DCM than in diabetic patients without cardiomyopathy and healthy controls, and no significant differences exists between diabetic patients without cardiopathy and healthy controls[144]. Intriguingly, HG treatment does not affect the expression of lncRNA-*TINCR* in human cardiomyocytes; however, lncRNA-*TINCR* overexpression inhibits cardiomyocyte apoptosis induced by HG treatment.

For instance, trimetazidine, an anti-ischemic (anti-anginal) metabolic agent of the fatty acid oxidation inhibitor class, was demonstrated to produce cardioprotective effects against apoptosis so as to attenuate the phenotypes of DCM[145]. Nicorandil, a vasodilatory drug used to treat angina, increase the level of NO in the serum and eNOS in the heart of diabetic rats compared with the untreated diabetic group, leading to inhibition of apoptosis likely *via* enhancing the PI3K/AKT signaling[117]. Ivabradine, a medication used for the symptomatic management of stable heart-related chest pain and HF not fully managed by beta blockers, was shown to yield antiapoptotic effects in streptozotocin-induced diabetic cardiomyopathy[146]. LCZ696 is an angiotensin receptor-neprilysin inhibitor in clinical use and it can ameliorate diabetic cardiomyopathy by inhibiting inflammation, oxidative stress and apoptosis[147]. The activation of the AKT−FOXO1 signaling pathway by mitoKATP plays an important role in improving cardiac function and inhibiting apoptosis in DCM. Opening of mitoKATP by diazoxide (DZX) improves cardiac function and inhibits apoptosis via the AKT−FOXO1 signaling pathway in diabetic cardiomyopathy[148.] Human recombinant relaxin-3 (H3 relaxin) is a novel bioactive peptide that inhibits cardiac injury. H3 relaxin protects against myocardial injury in experimental diabetic cardiomyopathy by inhibiting myocardial apoptosis, fibrosis, and inflammation[22]. Atorvastatin oral tablet is a prescription medication used to improve cholesterol levels and decrease your risk for heart attack and stroke. A study reported by Abdel-Hamid et al.[149] concluded that this compound preserves myocardial structure in DCM by inhibiting cardiac apoptosis.

Fisetin is a bioactive flavanol molecule found in many plants and possesses various biological activities. In diabetic rats that exhibited hyperglycemia, increased glycosylated hemoglobin and serum lipids accompanied with significant hypoinsulinism, excessive oxidative stress, inflammation, and apoptosis, treatment with fisetin suppressed oxidative stress, inflammation and apoptosis[150]. Curcumin is a yellow pigment found primarily in turmeric, a flowering plant of the ginger family best known as a spice used in curry and a polyphenol with anti-inflammatory properties and the ability to increase the amount of antioxidants that the body produces. It was observed that curcumin activated AMPK and JNK1, which phosphorylated BCL-2 and BIM and subsequently disrupted their interactions with Beclin1, thereby promoting autophagy and alleviating apoptosis. Evidence was obtained that AMPK-mediated inhibition of mTORC1 pathway likely plays a role in regulating autophagy by curcumin under diabetic condition[151]. A study described that treatment with crocin, a carotenoid chemical compound that is found in the flowers crocus and gardenia, improves cardiac dysfunction by normalizing autophagy and inhibiting apoptosis in STZ-induced diabetic cardiomyopathy[152]. Another study demonstrated that catalpol, an iridoid glucoside contained richly in the roots of the small flowering plant species *Rehmannia glutinosa* Libosch, attenuates cardiomyocyte apoptosis in DCM via NEAT1/miR-140-5p/HDAC4 axis[153]. A SIRT1 activator, resveratrol, was shown to reduce cardiomyocyte apoptosis and restore cardiac dysfunction by ameliorating ER stress[154].

The findings that DCM apoptosis can be effectively inhibited by some therapeutic agents and natural bioactive substances to improve cardiac function support the prospect that apoptosis is a viable cellular target for the clinical treatment of DCM and the associated cardiac remodeling leading to HF. Especially, studies have demonstrated that inhibition of caspase-3, an executioner of apoptotic cell death, alleviates or prevents DCM, which on one hand verifies the role of apoptosis in the development of DCM and on the other hand supports suppressing apoptosis as a therapeutic approach for the treatment of this cardiac complication of diabetes[155,156].

4. Autophagic cell death and autosis in diabetic cardiomyopathy

Autophagy has been controversial in the sense of whether it is beneficial or disadvantageous to the heart. On one hand, as a degradation process to eliminate damaged proteins and dysfunctional organelles, autophagy to a proper extent produces pro-survival effects, but excessive autophagy give rise to harmful impacts. In general, proper autophagy protects cardiomyocytes against apoptosis, whereas deviation from appropriate levels of autophagy can cause cellular dysfunction and autophagic cell death[24,122,124,125,157,158]. Excessive activation of autophagy induces autophagic cell death and autosis[23–25,75,77]. It appears that autophagy is a coin with double sides in terms of its effects in the heart, and there is a mechanism for the switch between beneficial autophagy and deleterious apoptosis. Evidence has been provided for the role of the AMP-activated protein kinase (AMPK) in regulating the autophagy−apoptosis switch. T2DM is characterized by suppression of AMPK signaling as well as an impairment of autophagy, which contributes to an increase in oxidative stress and the development of DCM[159,160]. A study documented by Zou's research group[124] demonstrated that in T1DM OVE26 mice, the AMP-activated protein kinase (AMPK) is reduced along with impaired cardiac autophagy and cardiac function. Consistently, genetic inhibition of AMPK in cardiomyocytes attenuates autophagy, exacerbates cardiac dysfunction and increases mortality of diabetic mice. Chronic AMPK activation with metformin, one of the most used antidiabetic drugs and a well-characterized AMPK activator, significantly enhances autophagic activity, preserves cardiac function and prevents most of the primary characteristics of DCM in OVE26 mice. Dissociation of BCL2 from BECN1 through activation of MAPK8−BCL2 signaling may be an important mechanism by which AMPK activation restores autophagy, protects against cardiac apoptosis, and prevents DCM[159,160]. It is commonly recognized that diabetes suppresses cardiac autophagy and promotes apoptosis, which is one of the key processes in the pathogenesis of DCM. Depression of AMPK activity, inhibition of MAPK8/JNK1−BCL2 signaling, and promotion of the interaction between BECN1 and BCL2 might be the underlying mechanisms for the suppression of autophagy in DCM. On the other hand, enhancement of autophagy by metformin ameliorates DCM and cardiac dysfunction[161,162].

Another line of evidence for the interplay between autophagy and apoptosis comes from the studies on the therapeutic agents and bioactive natural substances as described in the previous section[117,145–154]. While these compounds have anti-apoptotic properties, they also enhance autophagy in the setting of DCM.

Increasing lines of evidence suggest that cardiomyocytes can be killed by autophagy[163–165]. A study using mouse models with gain- and loss-of-autophagy reported that diminished autophagy limits cardiac injury in a mouse model of T1DM, suggesting that uncontrolled autophagy elicits detrimental effects presumably by causing autophagic cell death in their model[166]. Another study described that myocardial ischemia/reperfusion (MI/R) induced autosis in cardiomyocytes, especially at 6 h of reperfusion, which was accompanied by upregulation of Rubicon, attenuation of autophagic flux, and marked accumulation of autophagosomes[75]. Genetic knockdown of Rubicon inhibited autosis and reduced I/R injury. Suppression of autosis by ouabain, a cardiac glycoside, in humanized Na^+/K^+-ATPase-knockin mice reduced I/R injury[75].

It is known that sodium-glucose cotransporter 2 (SGLT2) inhibitors reduce cardiovascular mortality in patients with DM. A study demonstrated that a SGLT2 inhibitor, empagliflozin produces cardioprotective effects by suppressing cardiomyocyte autosis in myocardial infarction mouse models with and without DM. Empagliflozin treatment significantly reduces infarct size and improves cardiomyocyte survival through directly inhibiting the activity of the Na^+/H^+ exchanger 1. These beneficial effects are verified by *in vitro* and *in vivo* analysis of *Nhe1* and Beclin 1 knockout mice[167].

However, it should be noted that studies on autophagic cell death and autosis in DCM is still scant and direct evidence for the roles of these types of cell death in the development of DCM is still lacking.

5. Pyroptosis in diabetic cardiomyopathy

Inflammatory response is a common and critical pathological process in the development of cardiovascular disease including DCM, which often leads to pyroptosis, a non-apoptotic caspase-1-dependent pro-inflammatory programmed cell death[78–83]. Apart from apoptosis, pyroptosis has also been extensively characterized

and widely accepted as an alternative form of cell death in DCM. In addition to endogenous factors including pro-inflammatory proteins like caspase-1, cytokines, GSDMD and NLRP3, pyroptosis is also finely regulated by diverse signaling mediators and ncRNAs including miRNAs, lncRNAs and circular RNAs (circRNAs), as well as therapeutic drugs and bioactive natural substances used in clinics.

It is known that ROS-induced activation of NLRP3 inflammasome plays an important role in MI/R injury. A study investigated whether diabetes affects MI/R injury through NLRP3 inflammasome-mediated pyroptosis in T1DM rat model established by intraperitoneal injection of STZ and in H9C2 cardiomyocytes exposed to HG and subjected to hypoxia/reoxygenation (H/R) stimulation. The authors observed the increased NLRP3 inflammasome activation and pyroptosis in MI/R and HG and H/R, which is inhibited by inflammasome inhibitor BAY11-7082[168]. Evidence for pyroptosis was also obtained in T2DM rats by another group[169]. It was found that while silencing of NLRP3 *in vivo* does not affect systemic metabolic disturbances, it effectively ameliorates cardiac inflammation, pyroptosis, and cardiac function. Consistently, silencing of NLRP3 in H9C2 cardiomyocytes suppresses pyroptosis under HG. ROS inhibition markedly decreases nuclear factor-κB (NF-κB) phosphorylation, thioredoxin interacting/inhibiting protein (TXNIP), NLRP3 inflammasome, and mature IL-1β in H9C2 cells exposed to HG. The authors concluded that NLRP3 inflammasome contributes to the development of DCM in type IIDM rats.

Absent in melanoma 2 (AIM2) is a cytosolic DNA sensor which plays an important role in inflammasome formation. The results from a recent study suggests that AIM2 plays an important role in pyroptosis in HG-induced, ROS-mediated diabetic cardiomyopathy *via* the GSDMD pathway[170]. This study employed a rat model of T1DM and cultured H9C2 cells stimulated by HG. AIM2 expression was found significantly increased in diabetic myocardium compared with that in non-diabetic tissues, along with severe left ventricular dysfunction and cardiomyocyte death. Gene silencing of *Aim2* alleviates cardiac dysfunction in T1DM rats and GSDMD-N-related pyroptosis in H9C2 cardiomyoblasts.

Chemerin, a chemoattractant protein that acts as a ligand for the G protein-coupled receptor CMKLR1 and its receptor CMKLR1 (a G-protein-coupled receptor) are potent inducers of inflammation. Xie et al.[171] investigated the role of chemerin/CMKLR1 in mediating inflammation and regulating pyroptotic cell death in rats with T2DM. Their results showed that the expression of chemerin, CMKLR1, NLRP3, pro-caspase-1, activated caspase-1, and mature IL-1β was all upregulated in DCM with significant pyroptosis. Silencing of CMKLR1 *in vivo* attenuated the expression of NLRP3 and activated caspase-1 and IL-1β, accompanied by mitigated inflammation and pyroptosis, as well as improved cardiac function. The findings indicate the critical role of chemerin/CMKLR1 axis in diabetic cardiomyocyte pyroptosis.

ALDH2 is a conserved detoxifying mitochondrial enzyme aldehyde dehydrogenase 2, notably implicated in the metabolism of aldehydes. It was demonstrated that ALDH2 produces anti-pyroptotic action in H9C2 cells under HG to mimic hyperglycemic conditions[172]. Specifically, ALDH2 overexpression decreases the production of ROS and protein expression of NLRP3 inflammasome, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), caspase-1, and IL-18 and IL-1β. These effects protected H9C2 from HG-induced pyroptotic cell death. Yet, these results were obtained from *in vitro* experiments without validation in animal models of DCM. Diabetes aggravates MI/R injury because of the combination effects of changes in glucose and lipid energy metabolism, oxidative stress, and systemic inflammatory response.

Lipopolysaccharides (LPS), also known as endotoxins, are large molecules consisting of a lipid and a polysaccharide found in the outer membrane of Gram-negative bacteria. LPS was reported to counteract the increases in the expression of NLRP3, ASC, cleaved caspase-1, IL-1β, and IL-18, and NLRP3 inflammasome-mediated pyroptosis in the conditions of hypoxia followed by reoxygenation (H/R)[173]. A similar issue to that in the study on ALDH2 described above is that the results were acquired merely from H9C2 myoblasts and may not be applicable to the real situation of DCM.

Several studies unraveled that pyroptosis is a target cellular process for ncRNAs including miRNAs, lncRNAs and circular RNAs (circRNAs). A study revealed that miR-30d expression is substantially increased in STZ-induced T1DM rats and in high-glucose-treated cardiomyocytes as well[174]. Upregulation of miR-30d promotes cardiomyocyte pyroptosis in DCM; conversely, knockdown of miR-30d attenuates it with concomitant down-regulation of caspase-1 and pro-inflammatory cytokines IL-1β and IL-18. The signaling mechanisms underlying the pro-pyroptotic property of miR-30d is ascribed to the repression of FOXO3a expression and its downstream protein, apoptosis repressor with caspase recruitment domain ARC. According to the findings, the authors proposed a new signaling pathway leading to cardiomyocyte pyroptosis in DCM: miR-30d$\uparrow \rightarrow$FOXO3a$\downarrow \rightarrow$ ARC$\downarrow \rightarrow$caspase-1$\uparrow \rightarrow$IL-1β/IL-18$\uparrow \rightarrow$pyroptosis$\uparrow$.

The study documented by Jeyabal et al.[175] provided impressive and solid evidence for the presence of pyroptosis as a form of cell death in HG-treated human ventricular cardiomyocytes and in human diabetic hearts. This study also elucidated the critical roles of miR-9 and ELAV-like protein 1 (ELAVL1) in the inflammatory response and the associated pyroptosis as well as in the progression of HF induced by DCM. Specifically, their data demonstrated that ELAVL1 expression is augmented with a concomitant increase in caspase-1 and IL-1β expression in human hearts and human ventricular cardiomyocytes under hyperglycemic condition. ELAVL1 knockdown abrogates TNF-α induced canonical pyroptosis *via* NLRP3, caspase-1 and IL-1β suppression. Expression of miR-9 is significantly reduced in HG-treated cardiomyocytes and in human diabetic hearts, which results in upregulation of ELAVL1 expression and activation of caspase-1. In contrast, miR-9 mimics attenuates hyperglycemia-induced ELAVL1 and inhibits cardiomyocyte pyroptosis. The findings highlight the anti-pyroptotic action of miR-9 and potential therapeutic implication of targeting miR-9/ELAVL1 in preventing cardiomyocyte cell loss during HF in diabetics. miR-214 has also been established as an anti-pyroptotic miRNA in H9C2 cells cultured under hyperglycemic conditions[176]. miR-214 directly targets caspase-1 to antagonize pyroptotic cell death. Yet, the results remained yet to be verified by *in vivo* experiments.

miRNA can also mediate the regulatory effects of lncRNA on diabetic pyroptosis. A study from Wang's research team[177] showed that lncRNA-*Kcnq1ot1* is abnormally upregulated in the hearts of diabetic patients, diabetic mice, and HG-treated cardiomyocytes. Silencing lncRNA-*Kcnq1ot1* by a lentivirus-shRNA alleviates pyroptosis by targeting miR-214-3p and caspase-1, which is accompanied by reduced pyroptotic cell death, cytoskeletal structure abnormalities and calcium overload *in vitro* and improved cardiac function and morphology *in vivo*. LncRNA-*Kcnq1ot1* is overexpressed in DCM, and silencing lncRNA-*Kcnq1ot1* inhibits pyroptosis by influencing miR-214-3p and

caspase-1 expression. The same group subsequently reported that silencing of lncRNA-*Kcnq1ot1* rescues cardiac dysfunction, diminishes fibrogenesis, and ameliorates pyroptosis *via* inactivating the TGF-β1/SMADs pathway in diabetic mice[178].

lncRNA-*GAS5* was found downregulated in a mouse model of DCM and atrial cell line HL-1[179]. Replacement of lncRNA-*GAS5* improves cardiac function and relieves myocardial hypertrophy in DCM mice, along with downregulation of NLRP3, caspase-1, Procaspase-1, IL-1β and IL-18. Moreover, lncRNA-*GAS5* overexpression suppresses caspase-1 activity, LDH release and the levels of IL-1β, IL-18 in the HG-treated HL-1 cells. Intriguingly, lncRNA-*GAS5* acts by sponging miR-34b-3p, indicating that like miR-214-3p, miR-34b-3p also mediates the action of lncRNA. Unlike lncRNA-*Kcnq1ot1* that is a pro-pyroptotic lncRNA, lncRNA-*GAS5* appears to be an anti-pyroptotic lncRNA.

As another subclass of ncRNA, circular RNAs (circRNAs) also play important roles in regulating cardiac disease. The research team that first uncovered the role of lncRNA in regulating diabetic pyroptosis also identified for the first time that a circRNA named caspase-1-associated circRNA (circR-*CACR*) controls pyroptosis by sequestering miR-214-3p to increase caspase-1 thereby pyroptotic cardiomyocyte death in DCM[180]. The authors established circR-*CACR* as a ceRNA for endogenous miR-214-3p and then demonstrated that circR-*CACR* knockdown in cardiomyocytes suppresses high-glucose-induced caspase-1 activation.

Like apoptosis, pyroptosis is also modulated by some therapeutic drugs. Having established the role of pyroptosis in the development of DCM, Wang's team[177] further unraveled that metformin, a widely used antidiabetic drug for type II diabetes elicits a cardioprotective effects against pyroptosis. Their study revealed that in STZ-induced DM mice and HG-treated primary cardiomyocytes from neonatal mice, metformin decreases the expression levels of mTOR, NLRP3, caspase-1, IL-1β and GSDMD-N, and these beneficial effects were reversed by AMPK inhibitor. The data suggest that metformin can activate AMPK to improve autophagy *via* inhibiting the mTOR pathway and to suppress pyroptosis and the development of DCM.

Exendin-4, a peptide agonist of the glucagon-like peptide (GLP) receptor that promotes insulin secretion, has been shown to favor the survival of cardiomyocytes in DCM. A study showed that Exendin-4 protects against heart remolding and dysfunction and attenuates cardiac inflammation in DM rats induced by high-fat diet[181]. Exendin-4 significantly inhibits the activity of caspase-1 and production of pyroptotic cytokines in the diabetic heart and in HG-treated cardiomyocytes as well. Inhibition of AMPK activity mitigates the anti-pyroptotic property of Exendin-4, indicating that AMPK activation underlies the beneficial action of Exendin-4.

6. Necroptosis in diabetic cardiomyopathy

The role of necroptosis in DCM is largely unclear, since the relevant investigations have been rather sparse. To date, there have been only one report with an animal model and a few studies carried out *in vitro* in simulated DCM with cultured cells.

The *in vivo* study reported by Song et al.[182] used STZ-induced T1DM mice and neonatal rat cardiomyocytes cultured under HG conditions to investigate the role of SIRT3 in DCM. The authors first demonstrated that SIRT3 expression in the myocardium of diabetic mice was lower than that of control counterparts, using proteomics analysis in conjunction with real-time PCR and Western blot analysis. They then observed that SIRT3-deficient diabetic mice exhibited aggravated cardiac dysfunction, increased lactate dehydrogenase (LDH) level in the serum, decreased ATP level in the myocardium, exacerbated myocardial injury, and enhanced myocardial ROS accumulation. Similar changes were seen in neonatal rat cardiomyocytes exposed to HG. Notably, SIRT3 deficiency upregulated the expression of necroptosis-related proteins including RIPK1, RIPK3, cleaved caspase-3, inflammation-related proteins including NLRP3, caspase-1 P20, and interleukin-1β both *in vitro* and *in vivo*.

A recent study aimed to explore the cytoprotective role of inhibitor 1 of protein phosphatase 1 (I1PP1) in HG-induced cardiomyocyte injury presented a piece of evidence for the presence of necroptosis in cardiomyocytes[183]. The results showed that HG elevated the protein levels of RIPK3 and cleaved caspase-3 and decreased ATP level, oxidative stress, and mitochondrial membrane potential, leading to necroptotic cardiomyocyte death. In contrast, I1PP1 overexpression alleviates these deleterious alterations.

Another report described that exposure to HG suppresses cell viability and downregulates the expression of ALDH2 at the mRNA and protein levels but promotes ROS production and the expression of necroptosis-related genes RIP1, RIP3, and MLKL in H9C2 cells[184]. Necrostain-1 (a specific inhibitor of necroptosis) or ALDA-1 (ALDH2 activator) attenuates HG-induced downregulation of ALDH2 and upregulation of RIP1, RIP3 and MLKL. The findings indicate the role of necroptosis in simulated DCM.

Still another investigation explored the potential contribution of inactivation of the ROS-TLR4-necroptosis pathway to the cytoprotective effect of ATP-sensitive K^+ (K_{ATP}) channel opening against HG-induced cardiomyocyte injury[185]. It was found that HG increases the expression of Toll-like receptor 4 (TLR4) and RIP3, whereas inhibition of necroptosis by necrostatin-1 (an inhibitor of necroptosis) or by necrostatin-1 along with TAK-242 (an inhibitor of TLR4) attenuates the upregulation of RIP3. On the other hand, pre-treatment with diazoxide (a mitochondrial K_{ATP} channel opener) or pinacidil (a non-selective K_{ATP} channel opener) or *N*-acetyl-L-cysteine (a ROS scavenger) prevents the upregulation of TLR4 and RIP3, decrease in cell viability, and increases in ROS generation, MMP loss, and inflammatory cytokines secretion.

Direct evidence for the presence and significance of necroptosis in DCM is still lacking, and so is the specific timing of necroptosis occurrence during the development of DCM. Thus, at present it remains unclear whether necroptosis is a consequence or a cause of DCM.

7. Ferroptosis in diabetic cardiomyopathy

Ferroptosis is a new form of regulated cell death which is triggered by Fe^{2+}-dependent lipid peroxidation with an insufficient cellular reducing capacity. Excessive production of intracellular iron can cause ferroptosis by promoting ROS generation through Fenton reaction on various lipid membranes in cell[96–101]. Ferroptosis has been claimed to participate in DCM by two reported studies[35,36].

One of these two studies was performed in the setting of myocardial I/R injury in streptozotocin-induced T1DM rats and

hypoxia reoxygenation (H/R) injury in cultured H9C2 cardiomyoblasts, as well as non-DM animal for control[35]. The results show that both myocardial I/R injury and cardiomyocyte H/R injury induce ferroptosis and ER stress, and these pernicious alterations are aggravated in DM hearts and HG-treated H9C2 cells relative to non-DM hearts and cells cultured in normal medium, respectively. Inhibition of ferroptosis by Ferrostatin-1 ameliorates in MI/R injury in DM rats and in H/R injury in HG-stimulated H9C2 cells. In sharp contrast, ferroptosis activator Erastin exacerbates ER stress and ferroptotic cell death. On the other hand, inhibition of ER stress by salubrinal mitigates ferroptosis, whereas ER stress agonist tunicamycin exacerbates it. These findings suggest that in diabetic myocardium, ferroptosis is functionally coupled with ER stress.

In the second report, the investigators utilized palmitic acid (PA), the most common saturated fatty acid accounting for 20%−30% of total fatty acids found in the human body, to induced myocardial injury[36]. They found that PA induced the cell death in H9C2 cardiomyoblasts and primary neonatal rat cardiomyocytes in a dose- and time-dependent manner, which was substantially abrogated by varying ferroptosis inhibitors. Meanwhile, PA decreased the protein expression levels of heat shock factor 1 (HSF1) and GPX4 in a dose- and time-dependent manner, which was also restored by ferroptosis inhibitors. Moreover, overexpression of HSF1 alleviated PA-induced ferroptotic cell death and lipid peroxidation and improved disturbed iron homeostasis by regulating the transcription of iron metabolism-related genes (*e.g.*, *Fth1*, *Tfrc*, *Slc40a1*). Similarly, GPX4 overexpression protected against PA-induced ferroptosis, whereas knockdown of GPX4 reversed the anti-ferroptotic effect of HSF1. Consistent with the *in vitro* findings, PA-challenged $Hsf1^{-/-}$ mice exhibited more serious ferroptosis, increased *Slc40a1* and *Fth1* mRNA expression, decreased *Gpx4* and *Tfrc* expression, and enhanced ER stress in the heart, compared with $Hsf1^{+/+}$ mice. While these data are not directly linked to diabetes, chronic consumption of high fat diets rich in saturated fatty acids like PA is considered a critical contributor to the development of obesity and T2DM.

Similar to necroptosis, the evidence available to date for the role of ferroptosis in DCM was obtained mainly from *in vitro* studies with cells cultured in high glucose with only one animal study with T1DM model. Thus, while the existing studies support the possible presence of these forms of cell death in DCM, future studies with more solid experimental and clinical evidence are required before a conclusion can be reached as to whether they bear any pathophysiological significance and importance.

8. Conclusions and perspectives

Evidence is accumulating for the existence of multiple forms of cardiac cell death in DCM and the pathological consequences of these cell deaths to cardiac function. Probably most notable is the fact that in all *in vivo* animal studies published thus far, suppression of cell death of a whatever form comes up with appreciable restoration of impaired cardiac function and structure in DCM. These common findings ascertain cell death of any types as a promising cellular target for correcting the detrimental outcomes and perhaps also for retarding the degenerative progression of DCM. The relative importance of various forms of cell death identified by far in DCM induced by T1DM or T1DM and T2DM is schematically depicted in Fig. 2. Nonetheless, at present the pathophysiological roles of these cell deaths remain incompletely understood and the possible differences in the specific pathophysiological roles of different forms of cell death are even less elucidated. Due to still rather sparse studies and limited data available, the present review cannot get further insight into the mechanistic links between multiple forms of cell death and DCM, and thus many relevant questions remain unanswered. For example, what exact role does each form of cell death plays in the development of DCM? Do different forms of cell death occur at different stages of the development of DCM? Whether the occurrence of various forms of cell death depends the severity of glucotoxicity or lipotoxicity? Whether different forms of cell death contribute to the pathological mechanisms between T1DM and T2DM? Does one form of cell death affect others in the setting of DCM? Clearly, these issues merit future studies. The aim of this review article is to advocate and facilitate boarder and more in-depth research activities pertinent to cell death in DCM.

Apoptosis is the best characterized and well recognized form of cell death in DCM of both T1DM and T2DM with extensive and intensive investigations. Pyroptosis as a distinct mechanism of cell death has also been established, but how it contributes to the development of DCM remains yet to be delineated. Though there also exist some data in support of the other forms of cell death (autophagic cell death and autosis, necroptosis and ferroptosis) in DCM, solid and convincing experimental attestation is still currently lacking. For example, it is unascertained if autophagic cell death and autosis are actually operating in DCM despite that autophagy has been firmly established and the machinery for autophagic cell death and autosis are all present in this pathological condition. Similarly, while the experimental evidence for necroptosis and ferroptosis has been documented, scant studies using animal models of DCM (only one report for necroptosis or ferroptosis) and insufficiently strong data may not let us reach a conclusion on the pathophysiological significance and clinical implications of these forms of cell death in DCM. Clearly, we are merely in the early stage of our exploration on cell death in DCM and far from complete and in-depth understanding of the matter. Therefore, one of the future directions of the research field is to extend the investigations to other forms of cell death in addition to apoptosis and pyroptosis and to clarify the pathophysiological roles of alternative cell death in determining the development and consequence of DCM.

Apart from identification and characterization of different forms of cell death in the setting of DCM, it is of paramount importance to understanding how the regulated cell death of different forms is programmed and coordinated or whether different forms of cell death crosstalk one anther during DCM. Or more specifically, we need to know whether different forms of cell death occur simultaneously or at different stages of DCM. It is likely that various forms of cell death overlap at the same stage but have different contributions to DCM at different stages with some being more important to the development of DCM and others to the progression of DCM to HF. The ground for this speculation is indeed present in DCM characterized by co-existence of excessive ROS production and inflammatory response, which could well trigger both apoptosis and pyroptosis, and even the ferroptotic signaling pathways. Elucidating this issue should help us develop better strategies for precise targeting of a particular form of cell death to efficiently alleviate DCM and/or bock its degeneration to more severe conditions. Unfortunately, there has been no such studies in the literature to date.

The metabolic disturbance in diabetes generates a plethora of conditions/substrates, inducers/triggers, signaling mediators and

executioners leading to cell death of one or the other forms. In theory, interfering any of these steps should rescue cell death. In reality, however, it remains unresolved which is the optimal target for generating the best cardioprotective effects to prevent, slow or reverse the development of DCM and the progression of DCM into HF. This is another issue worthy of future efforts to clarify.

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Author contributions

Lihong Wang designed the work. Jinjing Wei wrote the paper under the guidance of Lihong Wang. Yongting Zhao, Haihai Liang, and Weijie Du revised the paper. Lihong Wang commented and corrected the manuscript. All of the authors have read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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