

The mechanisms of graphene-based materials-induced programmed cell death: a review of apoptosis, autophagy, and programmed necrosis

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Abstract: Graphene-based materials (GBMs) are widely used in many fields, including biomedicine. To date, much attention had been paid to the potential unexpected toxic effects of GBMs. Here, we review the recent literature regarding the impact of GBMs on programmed cell death (PCD). Apoptosis, autophagy, and programmed necrosis are three major PCDs. Mechanistic studies demonstrated that the mitochondrial pathways and MAPKs (JNK, ERK, and p38)- and TGF- β -related signaling pathways are implicated in GBMs-induced apoptosis. Autophagy, unlike apoptosis and necroptosis which are already clear cell death types, plays a vital pro-survival role in cell homeostasis, so its role in cell death should be carefully considered. However, GBMs always induce unrestrained autophagy accelerating cell death. GBMs trigger autophagy through inducing autophagosome accumulation and lysosome impairment. Mitochondrial dysfunction, ER stress, TLRs signaling pathways, and p38 MAPK and NF- κ B pathways participate in GBMs-induced autophagy. Programmed necrosis can be activated by RIP kinases, PARP, and TLR-4 signaling in macrophages after GBMs exposure. Though apoptosis, autophagy, and necroptosis are distinguished by some characteristics, their numerous signaling pathways comprise an interconnected network and correlate with each other, such as the TLRs, p53 signaling pathways, and the Beclin-1 and Bcl-2 interaction. A better understanding of the mechanisms of PCD induced by GBMs may allow for a thorough study of the toxicology of GBMs and a more precise determination of the consequences of human exposure to GBMs. These determinations will also benefit safety assessments of the biomedical and therapeutic applications of GBMs.

Keywords: graphene based materials, cell toxicity, programmed cell death, mechanisms

Introduction

Graphene materials have various applications and are widely used in the biomedical field in tumor therapy,¹⁻³ diagnostics,^{4,5} bio-imaging,⁶⁻⁸ and drug delivery.^{9,10} The widespread use of graphene materials has prompted concerns about the possible human health and environmental safety impacts of these materials. GBMs present in biomedical and non-biomedical products have shown potential toxicity to human beings, animals, and cells.¹¹⁻¹³ Several reviews have summarized that GBMs resulted in various degrees of cell death.^{14,15} For example, GO and rGO treatments resulted in dose-dependent cell death in A549 cells and HUVEC cells, and the functionalization decreased cytotoxicity and cell death.^{16,17} Cell death is a signal that reflects the toxicity of GBMs,¹⁸⁻²⁰ and this review focuses on the mechanisms of GBMs-induced PCD.

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A systematic classification of cell death was recently presented in a set of recommendations by the Nomenclature Committee on Cell Death.²¹ PCD is proposed to be death of a cell in any pathological format, when mediated by an intracellular program. PCD balances cell death with survival of normal cells when the equilibrium becomes disturbed. It is suggested that PCD is mediated by specific cellular mechanisms and that several signal pathways are activated and involved in these processes.²² Several PCD modalities, including apoptosis, autophagy, and programmed necrosis (also called necroptosis), have been investigated in the studies of the toxicity of nanomaterials.^{23,24} In particular, apoptosis, autophagy, and necroptosis are usually involved in GBMs toxicology.^{25–27} Many studies have reported that the different physicochemical properties of GBMs result in various cellular toxicities, and GBMs induce cell death in dose- or time-dependent manner.²⁸ However, the detailed mechanism of PCD induced by GBMs has not been well elucidated. Since many other reviews had illustrated that the different physicochemical properties of GBMs largely impacted on the cell toxicity,^{29–31} we concentrate on summarizing the pathways of PCD induced by GBMs in this review, and leave aside the effect of administration route, administration dose, and the physicochemical properties of GBMs.

Notably, apoptosis, autophagy, and necroptosis can be distinguished by their unique morphological differences and physiological processes; however, the underlying signaling pathways are intricate and delicate.^{32,33} Evidence has illustrated that apoptosis and necroptosis are two manners of cell death, whereas autophagy can play either pro-survival or pro-death roles. Under some conditions, apoptosis, autophagy, and necroptosis pathways are somehow cross-linked with each other. Herein, we review some studies on GBMs-induced PCD and summarize part of the signaling pathways, and analyze some differences and correlation between PCDs.

GBMs induce apoptosis in cells

Apoptosis is one of the most important toxic features described by studies of GBMs toxicity. GBMs induce apoptosis both in vivo and in vitro, and the extent of apoptosis differs depending on the varying properties of GBMs, including surface area, layer number, lateral dimension, functional groups, and surface chemistry. Recently, hydrophilic GO showed cellular uptake resulting in apoptosis following cell morphological changes from an elongated to a spherical morphology; conversely, hydrophobic rGO was found to be mostly adsorbed at cell surface without

internalization at a dose of 20 mg/L.^{34–36} Graphene layers (10 µg/mL) induced apoptosis by activating caspase-3 in a time-dependent manner.³⁷ GO nanoribbons (100 µg/mL) and GO polyethylenimine (1.6 µg/mL) triggered severe apoptosis by translocating to the cell nucleus.^{38,39} However, polyvinyl pyrrolidone-coated GO and nano-GO coated with polyethylene glycol triggered weaker apoptosis compared to GO in a dose-dependent manner.^{40,41}

GBMs-induced apoptosis somehow promoted diseases. The cell cycle arrest and DNA fragmentation caused by GO could induce germline apoptosis, affecting gonad development at the concentration of 10 mg/L.⁴² GQDs may translocate to the brain and localize to the nucleus of the diencephalon, causing Parkinson's disease-like symptoms by inducing cell apoptosis and senescence.⁴³ Long-term and chronic exposure to GBMs may result in some diseases or do harm to the health.

However, numerous studies have demonstrated the therapeutic value of GBMs in cancer cells. GBMs, similarly to several anticancer compounds, exert their inhibitory effect on tumors by arresting the cell cycle at a specific checkpoint, activating apoptosis, or a combined effect of both processes.⁴⁴ In vivo, GO and rGO injections decrease the mass and volume of tumors, and in vitro, GO and rGO (50 µg/mL) treatments induce apoptosis by the upregulation of caspase-3 and membrane leakage.^{45,46} GQDs (100 µg/mL) induced apoptosis of U251 human glioma cells displaying morphological and biochemical characteristics, such as phosphatidylserine externalization, caspase activation, and DNA fragmentation.⁴⁷ Similarly, PEGylated silver nanoparticles coated with GQDs exerted a distinct antitumor effect at the level of 100 µg/mL by triggering apoptosis only in cancer cells without impacting the viability of normal cells.⁴⁸ Consequently, understanding of how to improve the antitumor effects and decrease the toxicity of normal cells may allow good use of GBMs in biomedicine.

Since apoptosis induced by GBMs emerges in many situations, we summarize some information to describe GBMs-induced apoptosis, such as the physiochemical properties and functionalization of GBMs, the exposed cells, the exposed dose and time, and so on. The information is collectively presented in Table 1.

The signaling pathways involved in GBM-induced apoptosis

Apoptosis, or type I PCD, is a gene-controlled cell death process maintaining cell homeostasis and is characterized by specific morphological changes such as cell shrinkage,

Table I GBMs induced apoptosis

GBMs	Physiochemical properties and functionalization	Cells exposed	Dose and time of incubation	Effects (apoptosis)	References
Graphene layers	Size of 100–110 nm	PC12 cell line	0, 1, 4, 8, and 16 µg/mL	Caspase-3 activation	37
Pristine graphene	Thickness of 3–5 nm Size of 500–1,000 nm	RAW 264.7 cells	24 h 5, 10, 20, 40, 80, and 100 µg/mL	Trigger apoptosis via activation of the mitochondrial pathway	62
GO/rGO	Thickness of 2–3 nm Size of 40 nm for both GO and rGO Thickness of 6 nm for GO and 7 nm for rGO	HepG2 cells	24 and 48 h 5, 25, and 50 mg/L	GO induced NADPH oxidase, ROS formation, and apoptosis-related genes expression	34
Protein corona-GO nanoribbons	Size of ~100 nm Thickness of ~1.0 nm	A549 cells	100 µg/mL 24 h	Induced cell apoptosis	38
Pristine GO	Size of ~230 nm	T lymphocytes	0–100 µg/mL	Induced ROS-dependent apoptosis through Bcl-2 pathway	39
GO-COOH	Thickness of 1.28 nm		24 h		
GO-PEI					
NGO	Size of 100–200 nm	Zebrafish embryos	500 pg 30 h	Induced apoptosis in a dose-dependent manner	41
NGO-PEG	Thickness of 1–1.5 nm				
GQDs	Size of ~56.6 nm Thickness of ~1.9 nm	U251 human glioma cells	0–200 µg/mL 24 h	Induced the phosphatidylserine externalization, caspase activation, and DNA fragmentation	47
GO	Size of GO ~325 nm and rGO ~540 nm	Human ovarian A2780 cancer cells	20–100 µg/mL	Caused membrane leakage and upregulation of apoptosis executioner caspase-3	45
rGO	Thickness of GO ~4.74 nm and rGO ~11.14 nm		24 h		
Ag-GQDs	520–700 nm	HeLa and DU145 cancer cells	100 µg/mL 24 h	Induced apoptosis exhibiting a strong antitumor activity in cancer cells	48

Abbreviations: GBMs, graphene-based materials; GO, graphene oxide; rGO, reduced graphene oxide; PEI, polyethylenimine; NGO, nano-graphene oxide; PEG, polyethylene glycol; GQDs, graphene quantum dots.

nuclear condensation and fragmentation, and the appearance of apoptotic bodies.^{49,50} Apoptosis is divided into extrinsic (death receptor pathway) and intrinsic (mitochondrial pathway) pathways.⁵¹ The extrinsic pathway is mediated by the TNF receptor superfamily, and the binding ligands are Fas ligand, TNF-R, or TRAIL.⁵² The Fas/Fas-L complex recruits the FADD and pro-caspase-8 to form the DISC. This protein complex serves as a platform for caspase activation, and the autocatalytic activation of caspase-8 at the DISC leads to the proteolysis of pro-caspase-3 into active caspase-3, triggering the apoptotic process.⁵³ The intrinsic pathway is under the control of mitochondrial proenzymes with a variety of functions, including the dissipation of the mitochondrial transmembrane potential and the release of proapoptotic proteins into the cytosol, as well as a second, mitochondria-derived activator of caspases. After being triggered by extracellular stimuli or intracellular signals, internal cytochrome c is released into the cytosol from the permeable mitochondrial membranes and recruits Apaf-1 and pro-caspase-9 to the apoptosome, which activates the

downstream caspase-9/3 signaling cascade and consequently results in apoptosis.^{54,55}

Apoptosis is a complex process that can be activated through several signaling pathways.⁵⁶ Recently, much attention has been paid to clarifying the intricate mechanism of apoptosis in GBMs-induced toxicity. GBMs-induced apoptosis usually has the fundamental features of caspase activation, DNA fragmentation, increased oxidative stress, and calcium efflux.^{57,58}

Mechanistic studies demonstrated that mitochondria are implicated in apoptotic cell death, which act as major control points responsible for regulating apoptosis. It is well accepted that mitochondria participate in the intrinsic pathway of apoptosis and that they release soluble proteins from the intermembrane space to the cytosol to initiate caspase activation.^{59,60} For example, caspase-3, one of the most important executioner caspases, and PARP are activated by mitochondrial pathways that initiate pristine graphene-mediated apoptosis.^{61,62} Exposure to graphene increases intracellular ROS generation, including mitochondria-produced

superoxide, which subsequently results in the loss of the MMP.^{62,63} Disruptions of the MMP may lead to broken electron transfer chain, decline in ATP synthesis, and release of proapoptotic molecules from the mitochondria to cytoplasm. Similarly, GO and rGO treatments also cause mitochondrial damage and accelerate apoptosis by generating oxidative stress.^{64,65} Exposure to few-layer graphene caused elevation of cytoplasmic Ca^{2+} concentration and mitochondrial membrane depolarization, and eventually, the abnormal opening of the mitochondrial permeability transition pore led to more severe mitochondrial damage and the initiation of apoptosis.⁵⁷ Notably, TLRs also contribute to the loss of MMP, consequently inducing apoptosis after exposure to rGO.⁶⁶

In addition to mitochondrial pathways, the MAPKs (JNK, ERK, and p38)- and TGF- β -related signaling pathways are also involved in GBMs-induced apoptosis.⁶² The MAPK (JNK, ERK, and p38) signaling pathway involves a family of serine/threonine protein kinases that regulates cell proliferation, cell survival, and apoptosis.^{67,68} JNK activates the Bcl-2 protein family proapoptotic members, Bim and Bax, eliciting

MOMP during GBM-induced apoptosis.^{69,70} Subsequently, mitochondrial proapoptotic factors activate caspase-3, an important effector caspase involved in apoptosis.⁷¹ The p38 protein is activated by ROS and can induce apoptosis following the stimulation of TNF- α , the recruitment of caspase-8 and caspase-3, and the activation of the downstream effector protein PARP. In addition, TGF- β participating in cell apoptosis has been shown in several cell lines;^{72–74} however, the TGF- β -related signaling pathways have not been well studied in GBM-induced apoptosis. The proteins downstream of TGF- β , Smad proteins, activate proapoptotic factors and relocate from the mitochondria to the cytosol to regulate cell apoptosis. These pathways may be a good research direction for further study of GBMs-induced apoptosis. GBMs-induced apoptosis pathways are important parts of GBMs-induced toxicity, and some of them are delineated in Figure 1.

GBMs induce autophagy in cells

In the current toxicology study of GBMs, there is no detailed research on distinguishing between autophagy, autophagic

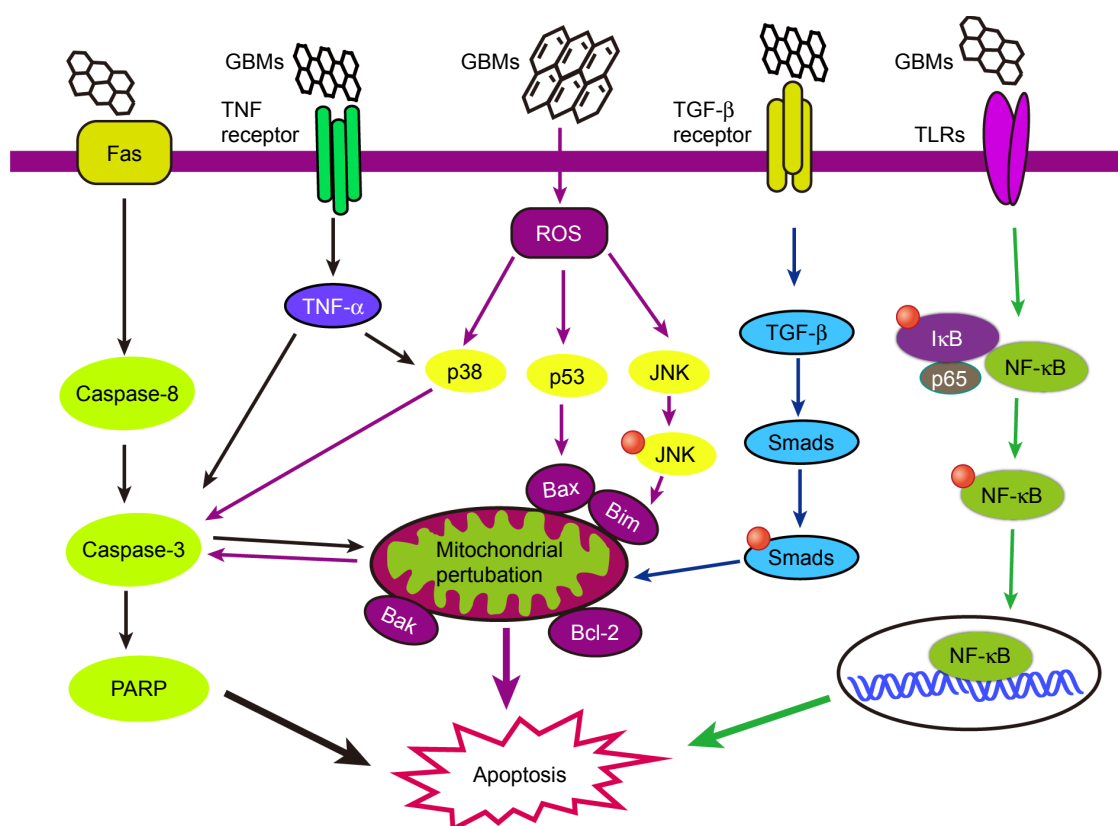


Figure 1 The signaling pathways involved in GBM-induced apoptosis. The schematic diagram delineates the extrinsic (death receptor pathway) and intrinsic (mitochondrial pathway) pathways of apoptosis. GBMs may induce apoptosis through mitochondrial pathways and the MAPKs- and TGF- β -related signaling pathways. Mitochondria act as major control points involving in regulation of apoptosis. Caspases (caspase-3, caspase-8), PARP, and Bcl-2 protein family proapoptotic members (Bim and Bax) are activated and mitochondrial outer membrane permeabilization and the loss of mitochondrial membrane permeabilization occur, and are involved in the pathways during GBM-induced apoptosis.

Abbreviation: GBMs, graphene-based materials.

Table 2 GBMs induced autophagy in cells

GBMs	Physicochemical properties and functionalization	Cells	Dose and time of incubation	Effects (autophagy)	References
GO	Size of ~450 nm Thickness of <2 nm	Mouse CT26 colon carcinoma cell	5, 25, 50, or 100 µg/mL 18 h	GO can be phagocytosed and trigger TLR-4/TLR-9 signaling cascades	77
GO	Size of large GO ~2.4 µm Size of small GO ~350 nm Thicknesses of ~1.0–1.2 nm	RAW 264.7 cells	5 or 100 µg/mL 24 h	Provoked TLR signaling cascades and triggered cytokine responses	75
GO	Size of ~300 nm Thickness of ~0.945 nm	Human–hamster hybrid AL cells	10 µg/mL 4 h	Triggered a genuine autophagic process	76
GQDs	Size of ~1.5 and 5.5 nm Thickness of ~1 nm	THP-1 monocyte cell line	10, 50, 100, and 200 µg/mL 24 h	Increased the expression of LC3-I/II and Beclin-1 in macrophages	26
Graphene nanoplatelets	Size of <2 µm Thickness of ~3–4 nm	BEAS-2B cells	2.5, 5, 10, and 20 µg/mL 24 h	Increased conversion from LC3B-I to LC3B-II, and the levels of ATG5, and Beclin-1 did not change	79
GO/CDDP	Size of ~450 nm Thickness of <2 nm	CT26 cells	50 µg/mL 24 h	Diverted the LC3 flux in the early phase of autophagy	78
GQDs	Size of ~60 nm Thickness of 2.3 nm	U251 human glioma cells	200 µg/mL 24 h	Formation of autophagic vesicles, LC3-I/LC3-II conversion, and degradation of autophagic target p62	47

Abbreviations: GBMs, graphene-based materials; GO, graphene oxide; GQDs, graphene quantum dots.

cell death, and cell death caused by autophagy. Rather, we just discuss autophagy triggered or inhibited by GBMs in the following text. GO triggers autophagy in macrophages in a concentration-dependent manner. Furthermore, GO can induce autophagy in various cell lines, including SNU-449, Mahlavu, A549, HEK293, and RAW 264.7 macrophages, at a concentration of 100 µg/mL by stimulating the TLR signaling cascades.⁷⁵ GO exposure to the human–hamster hybrid (AL) cells significantly elicited genuine autophagy by increasing the level of LC3-II and the autophagic flux even at a low dose (1 µg/mL) or an early time point (4 h).⁷⁶ GO was phagocytosed by murine colon carcinoma (CT26) cells and simultaneously triggered autophagy by TLR-4/9 signaling cascades at a dose of 50 µg/mL.⁷⁷ It was also suggested that GO was a potent autophagy inducer in CT26 colon cancer cells, and thus in chemotherapy, GO combined with the chemotherapy drug cisplatin (CDDP) (GO/CDDP) diverted the autophagic flux and improved antitumor effects at a dose of 50 µg/mL.⁷⁸ GNPs promoted autophagy by increasing the conversion from LC3B-I to LC3B-II and the level of p62 protein at 24 h after exposure in a dose-dependent manner in BEAS-2B cells.⁷⁹ Similarly, GQDs also significantly dose-dependently increased ROS generation and induced autophagy with an increase in the expression of LC3-II/I and Beclin-1 in macrophages or U251 human glioma cells.^{26,47} Since autophagy may eliminate GBM particles or provide energy through cleaning the remnant organelle in cells, it may become a primary index to evaluate the toxicity of GBMs in occupational exposure or biomedical application.

A summary of GBMs' physicochemical properties, exposed cells, toxicokinetics, and the autophagy results from the literature reviewed here is listed in Table 2.

The signaling pathways involved in GBMs-induced autophagy

Autophagic cell death is sometimes called type II PCD, and some experts had come up with an opposite opinion that autophagy might not cause cell death, and autophagy is different from autophagic cell death. They said that autophagic cell death is the cell death caused by autophagy rather than the cell death that autophagy participates in. Moreover, they pointed out that if the inhibitor of autophagy cannot prevent cell death, it cannot be called autophagic cell death.⁸⁰ Until now, the underlying mechanism of autophagy is far from clear. In addition, in the study of toxicity induced by GBMs, there are no detailed debates about whether autophagy can be called type II PCD. Furthermore in most situations, autophagy is taken as one kind of cell death or the main reason of cell death. GBMs always induced unrestrained autophagy, which would accelerate rather than prevent cell death.

Autophagy is characterized by the formation of autophagosomes, with double membrane-bound structures wrapping organelles and cytoplasmic macromolecules that subsequently fuse with lysosomes to form autolysosomes, thereby degrading the contents of the encapsulated vacuole.^{81,82} mTOR is the checkpoint at the initiation step of autophagy. Autophagy is initiated by negatively regulating the PI3K/Akt pathway and mTOR activity; however, Bcl-2 can bind to Beclin-1

and reverse this outcome. Moreover, the activated AMPK can induce autophagy through a phosphorylation event that stimulates TSC1/TSC2 activity, and the TSC1/TSC2 complex can negatively regulate mTORC1. In contrast, the phosphorylation and inactivation of TSC1/TSC2 allow for mTOR activation via the PI3K/Akt signaling pathway inhibiting autophagy.^{83–86} Many signaling pathways of autophagy have been verified in diseases, but those pathways need to be further studied in biocompatibility and toxicology of GBMs.

The classic process of autophagy is autophagosome accumulation and LC3-I/LC3-II conversion. Nascent LC3 is modified at its c-terminal domains by Atg4 and becomes LC3-I, which subsequently couples with phosphatidylethanolamine to become LC3-II. LC3-II then translocates to the autophagosome membrane,^{87–89} and the short hairpin RNA against LC3-II can block the autophagic response.⁴⁷ GO induced autophagosome accumulation and lysosome impairment disrupting intracellular homeostasis and promoted cell death after GBMs treatment in primary murine peritoneal macrophages and other cell lines.^{90,91} GO overload on lysosomal vesicles caused lysosomal membrane destabilization and significant lysosome fusion, which consequently resulted in the blockade of autophagosomes and autophagosome accumulation.⁸¹ It was elicited that lysosomal membrane permeabilization, lysosomal membrane rupture, and cytoskeleton disruption might be involved in lysosomal membrane destabilization, eventually resulting in autophagosome accumulation.⁹² GO also leads to a decrease of p62, a protein that directly binds to LC3 and selectively incorporates into autophagosomes.⁹³ Moreover, p62 is preferentially degraded by autolysosomes; this degradation process is inversely correlated with autophagosomal maturation and degradation.⁹⁴ However, a study reported that GO inhibited the maturation of autolysosomes and prevented the fusion between autophagosomes and lysosomes, thereby reducing the integrity of autophagic flux.⁹⁵ GBMs led to different consequences of autophagy depending on the various concentrations, exposure times, and particle sizes.

Mitochondrial dysfunction and ER stress play vital roles in GBMs-induced autophagy. GBMs have been shown to induce mitochondrial damage and provoke autophagy in a human bronchial epithelial cell line (BEAS-2B cells).⁷⁹ ROS and NO generation after GNPs treatment resulted in decreased numbers of mitochondria, ER, and Golgi apparatus, and mitochondrial fission-initiated mitochondrial autophagy.⁹⁶ GNPs dramatically increased the levels of p62 and Bcl-2 proteins to induce autophagy.⁷⁹ The adaptor protein p62 can bind to ubiquitinated mitochondrial proteins and LC3

on autophagosomes, recruiting autophagic membranes for mitochondrial clearance.⁹⁷ Furthermore, Bcl-2 and Bcl-xL are antiapoptotic proteins that bind to Beclin-1, and the dissociation of the Bcl-2–Beclin-1 complex is essential for the initiation of autophagy.⁹⁸ Moreover, the dissociation of the Bcl-2–Beclin-1 complex allows Beclin-1 to be activated by AMBRA1, a protein that may reside on mitochondria and bind to Bcl-2 until it is released by autophagic stimuli.⁹⁹ Autophagy selectively removed mitochondria and adjusted the number of mitochondria to supply energy, adapting to the cell survival environment after GBMs exposure.

Different TLRs such as TLR-2, TLR-3, TLR-4, and TLR-7 are proposed as autophagy inducers,^{100–102} and some TLR signaling pathways participate in GO-induced autophagy. GO-activated autophagy is mediated through TLR-4/9 signaling pathways associated with the downstream signaling proteins MyD88, TRAF6, and NF- κ B.⁷⁵ GO binds to TLRs activating MyD88 or TRIF, and recruiting the downstream proteins TRAF6 and Beclin-1. Beclin-1 can interact with TRAF6 to accelerate its activation and the formation of autophagosomes.^{101,103} Similarly, GO can be phagocytosed by CT26 colon cancer cells, triggering autophagy by both TLR-4 and TLR-9 signaling cascades. TLRs transduce signals first by MyD88, followed by TRAF6, NF- κ B, and IRF7, eventually resulting in various cellular responses including secretion of cytokines TNF- α , IL-1 β , and IFNs.⁷⁷ Except for TLR-4/9, the other TLRs have not been well studied in GBM-induced autophagy. It can be forecasted that the other TLRs may attract a lot of attention in the future.

Other signaling pathways are also involved in GBMs-induced autophagy. The p38 MAPK and NF- κ B pathways participate in GQD-induced autophagy in THP-1 macrophages. GQDs significantly increase the phosphorylation of p38 MAPK and p65 and promote NF- κ B translocation to the nucleus.²⁶ The overexpression or activation of p38 α disrupts the p38–mAtg9 interaction and thereby influences LC3 lipidation and p62 degradation, thus controlling the induction of autophagy.¹⁰⁴ Moreover, mTORC1 is a major checkpoint in autophagy, and the activation or inhibition of mTOR pathway has been studied in other diseases or nanomaterials that may be investigated in future studies of GBM-induced PCD. Some pathways of GBM-induced autophagy are demonstrated in Figure 2.

GBMs induce necroptosis and relative pathways involved

There are fewer studies of GBM-induced necroptosis compared to apoptosis and autophagy, and the mechanisms of

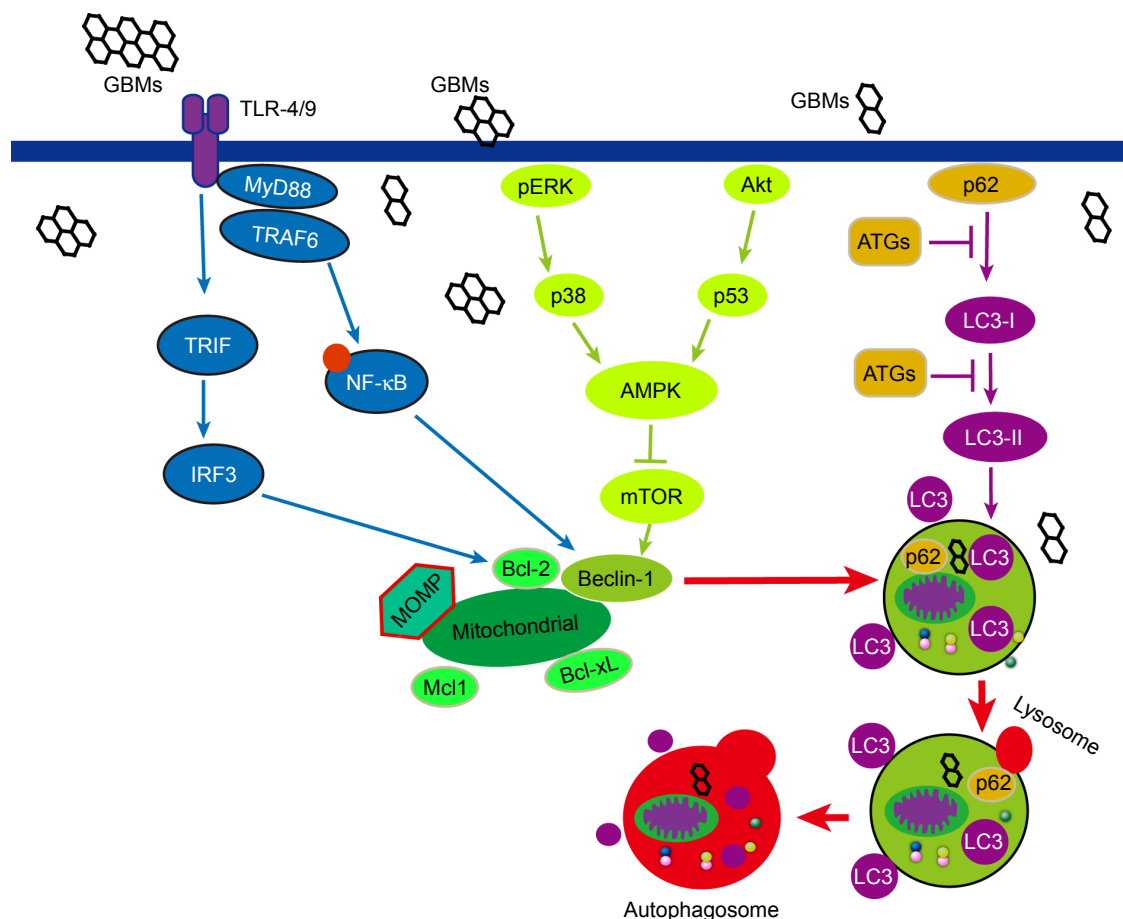


Figure 2 The signaling pathways involved in GBM-induced autophagy. The classic process of autophagy is autophagosome accumulation and LC3-I/LC3-II conversion which occurs after GBM treatment in murine peritoneal macrophages and other cell lines. Mitochondrial dysfunction and ER stress, TLRs, p38 MAPK, and NF- κ B pathways participate in GBMs-induced autophagy. mTORC1 is a major checkpoint in autophagy, and Beclin-1 connects autophagy with apoptosis.

Abbreviations: GBMs, graphene-based materials; MOMP, mitochondrial outer membrane permeabilization.

necroptosis are not well studied. With the discovery of key regulators of necrotic death, such as RIP kinases and PARP, the concept of programmed necrosis has recently gained ground. RIP3 phosphorylation forms a tight RIP1–RIP3 complex, which is often needed to initiate the necrotic programme.¹⁰⁵ The results of previous studies have demonstrated that GO/CDDP potentiates CT26 cell death through programmed necrosis when the cells are exposed to a concentration of 50 mg/mL for 24 h. GO/CDDP induces programmed necrosis by increasing the levels of RIP1, RIP3, and HMGB1, a nuclear protein that is released into cytosol and then released from cells after the initiation of necrosis.⁷⁸

Programmed necrosis, type III PCD, is a crucial type of cell death for the preservation of tissue homeostasis, the elimination of damaged cells, and immune responses.^{106,107} Signs of programmed necrosis include cell swelling, organelle dysfunction, and cell lysis.^{108,109} Although necrosis has always been considered an accidental form of cell death, the concept of programmed necrosis has been universally

recognized since the discovery of key mediators of programmed necrosis, such as RIP kinases and PARP.⁵³ The death receptor (TNFR1) and TLRs can induce RIPK1- and RIPK3-dependent necrosis in the presence of FADD, caspase-8, TRADD, and TIR-related adaptor proteins. Furthermore, calcium-activated calpain can invoke JNK resulting in necroptosis.^{110,111}

Programmed necrosis can also be activated by TLR-4 signaling in macrophages. GO (40 μ g/mL)-induced programmed necrosis in macrophages occurs through a TLR-4-dependent signaling pathway and is partially TNF-dependent; this latter process also involves RIP-dependent and RIP-independent signaling pathways.²⁷ GO disrupts the integrity of the cytoskeleton of macrophages and promotes ROS generation, which is critical to TLR-4-mediated macrophagic necrosis. Blocking TLR-4 signaling with a selective inhibitor or using TLR-4-deficient BMDMs leads to resistance against GO-triggered necrosis. Moreover, GO increases TNF- α secretion upon activation of the TLR-4 pathway, and

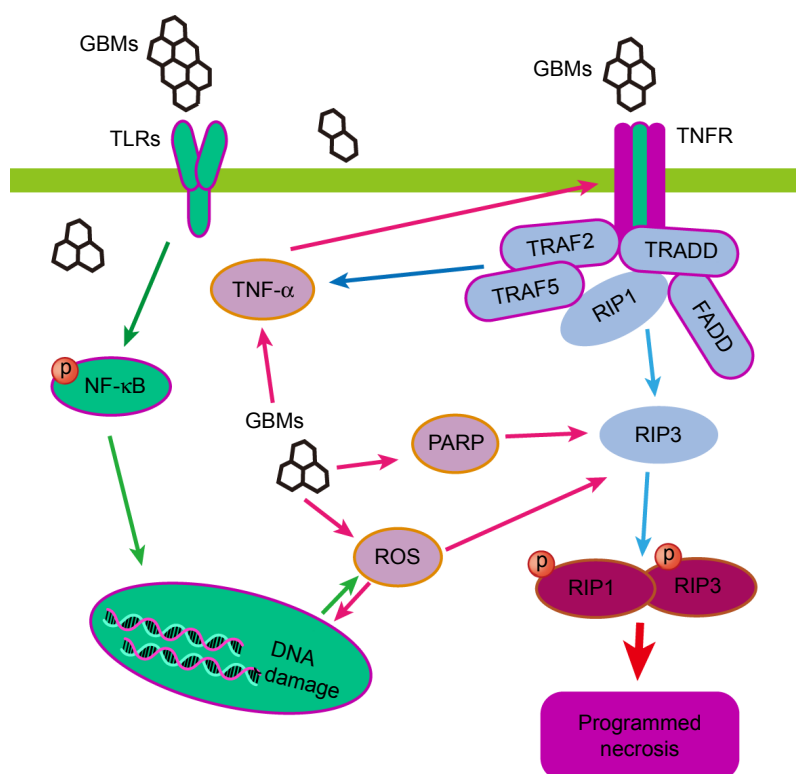


Figure 3 The signaling pathways involved in GBMs-induced necroptosis. RIP kinases and PARP are key regulators of programmed necrotic death, and the tight RIP1–RIP3 complex can initiate the necrotic program. GBMs can induce programmed necrosis through RIP-dependent and RIP-independent signaling pathways in macrophages via a TLR-4-dependent manner or partially by a TNF-dependent manner.

Abbreviation: GBMs, graphene-based materials.

TNF- α triggers programmed necrosis via the activation of the kinases RIP1 and RIP3 or other signaling pathways. The study of the mechanism underlying GO-mediated programmed necrosis is just a good beginning, the outcome the relative mechanism is far from clear and complete. Other programmed necrosis pathways involved in the diseases have not been discussed in the study of GBMs. Some of the pathways of GO-triggered programmed necrosis are displayed in Figure 3.

Some differences and relationships of GBMs-induced PCDs

Differences in PCD

From the discussion above, we find that we cannot sum up the law that GBMs induced what kinds of PCDs under certain circumstance. Different modes of PCDs appear in the same cells, which may be related to the cell phenotype, the physicochemical property and exposure time of GBMs, etc.¹¹² Often, several kinds of PCDs coexist in the same cell; however, we have not yet studied which kinds of PCDs come first in research of GBMs, nor do we try to understand how

GBMs induce several PCDs and maintain the balance. All of these queries may be answered in the future.

PCDs are distinguished by some characteristics. Apoptosis is morphologically characterized by cell shrinkage, chromatin aggregation, DNA fragmentation, and apoptotic body formation. In apoptosis, the early degradation of cytoskeleton occurs, but organelles degradation is preserved until the later stages. By contrast, autophagy occurs in the absence of chromatin condensation with massive autophagic vacuolization in the cytoplasm.^{81,113} Thus, the early disruption of organelles occurs, but the cytoskeleton is preserved until the late stage in the process of autophagy. Necroptosis is another morphologically and mechanistically distinct modality of cell death, characterized by mitochondrial lysis and lysosomal membrane rupture. In contrast to apoptosis and autophagy, necroptosis is characterized by a significant inflammatory response.¹¹⁴ Furthermore, unlike apoptosis which occurs in a caspase-dependent manner, necroptosis is caspase-independent. The phosphorylation and formation of RIP1 and RIP3 which only occur in necroptosis are specific indicators of necroptosis.^{105,115}

Several cross-linked pathways in PCD

Although apoptosis, autophagy, and necroptosis have different characteristics, their signaling pathways have certain correlations with each other. For instance, the suppression of apoptosis can trigger autophagy,¹¹⁶ while the absence of the caspase signaling pathway can trigger programmed necrosis; however, a specific inhibitor of programmed necrosis, Nec-1, can revert the death pathway to apoptosis.⁵³ From studies of other diseases, we conclude that the same signaling pathway plays different roles in different PCD processes and that different signaling pathways cross-link with each other to activate the same downstream effector molecules.

Biologists have investigated how apoptosis, autophagy, and necroptosis are interconnected.⁵³ Apoptosis and autophagy are two primary PCD mechanisms that interact with each other and often occur in the same cells.¹¹⁷ Apoptosis and autophagy can share key molecular regulators, such as p53, Akt, JNK, and Beclin-1.¹¹⁸ Apoptosis can suppress autophagy partly via the caspase kinase-mediated cleavage of essential autophagy proteins.¹¹⁹ Autophagy stimulates apoptosis by depleting endogenous inhibitors or attenuates apoptosis by selectively reducing proapoptotic proteins.¹²⁰ Apoptosis, autophagy, and necroptosis are initiated by a combination of related death receptors and ligands. For example, apoptosis and necrosis appear to be regulated by different forms of caspase-8: a fully processed, heterotetrameric form of caspase-8 induces apoptosis; however, caspase-8 mutants and caspase-8 inhibition activate necroptosis in some cell lines.^{106,121} Autophagy can trigger or block necroptosis in response to starvation and antigen stimulation in several cell lines.^{21,122–124}

Oxidative stress is involved in the toxic effects of GBMs. The generation of ROS is considered the primary cause of cell death.^{31,34} The increased ROS generation leads to apoptosis and autophagy with an increase in the expression levels of apoptotic and autophagic effectors, such as caspase-3, caspase-9, Beclin-1, Bax, Bad, and LC3-I/II, after exposure to GBMs.^{26,27} ROS induces the loss of the mitochondrial membrane that potentially initiates both apoptosis and necrosis.^{125,126}

Numerous signaling pathways comprise an interconnected network of apoptosis, autophagy, and necroptosis, such as the TLRs, p53 signaling pathways, and the Beclin-1 and Bcl-2 interaction. It is known that apoptosis can be induced via TLR-2, TLR-6, and TLR-9 pathways accompanied by the downstream proteins NIK and I κ B kinase in GBMs-mediated toxicity.^{66,127–129} Interestingly, autophagy is elicited

through the TLR-4 and TLR-9 pathways accompanied by the downstream adaptor proteins, MyD88, TRIF, and TRAF6, which were activated in RAW 264.7 macrophage cells and CT26 colon cancer cells after exposure to GO.^{75,77} Moreover, programmed necrosis can also be triggered via the TLR-4 pathway, so TLR-4-deficient BMDMs or a selective TLR-4 inhibitor CLI-095 constrains the effects of GO triggering programmed necrosis.²⁷ p53, a vital regulator of apoptosis, can modulate the MOMP and thereby govern the Bcl-2 family to trigger apoptosis.¹³⁰ In addition, p53 negatively controls autophagy through the AMPK/mTOR-dependent pathway by inhibiting mTOR via the activation of AMPK or the transactivation of DRAM.^{131,132} p53 is also involved in the regulation of necrosis.^{133–136} The functional and structural interactions between Beclin-1 and the antiapoptotic proteins Bcl-2 and Bcl-xL participate to some degree in the cross-linking of apoptosis, autophagy, and necroptosis.^{98,106,137,138}

Collectively, studies have indicated that apoptosis, autophagy, and necroptosis are independent but interconnected by similar pathways and share initiator or effector molecules. A part of the interrelationship of apoptosis, autophagy, and programmed necrosis is shown in Figure 4.

Summary

After summarizing and analyzing hundreds of articles on the toxic study of GBMs, we found that one or two or more kinds of PCDs occur simultaneously or successively in the same cells, and different types of cell deaths emerge simultaneously or successively depending on the physicochemical property, exposure dose, and exposure time of GBMs (these factors are not the focus of this review). The molecular mechanisms of PCD are multifaceted, complex, and interconnected, and the mechanism of PCD induced by GBMs is far from clear and complete. Understanding the mechanisms of PCD and relationships between apoptosis, autophagy, and programmed necrosis may benefit to figure out further the mechanism of GBMs-induced toxicity. The cognition of autophagy and apoptosis was limited by the experimental conditions and experimental methods years ago. The experimental techniques are improving over time, and our cognition of PCD is improving. Some original conclusions may need to be revised, redefined, and changed. Studies of GBMs-induced necroptosis are limited, and hence, more concerns are needed.

The role of autophagy in cell death should be carefully considered. Some researchers believed that autophagy may more likely be a kind of regulation mechanism, related to

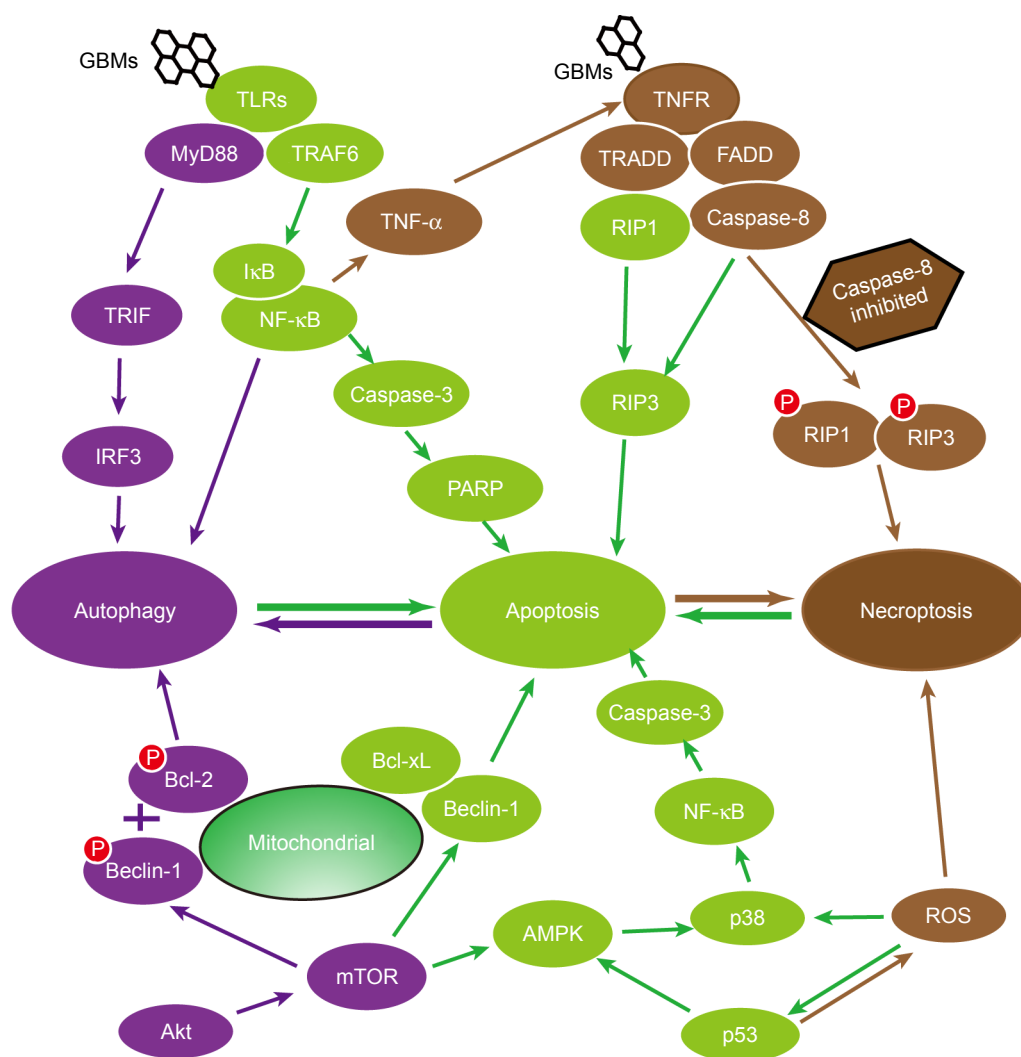


Figure 4 The interrelationships between programmed cell death. The schematic diagram depicts that apoptosis, autophagy, and necroptosis are independent but interconnected. They are often mediated by similar pathways, such as the TLR signaling pathway, the Beclin-1 and Bcl-2 interaction, and the p53 signaling pathway, and share initiator or effector molecules, comprising a complicated and delicate network.

Abbreviation: GBMs, graphene-based materials.

apoptosis or necrosis pathway components. The instinct of autophagy to remove the damaged proteins and organelles is of great significance for cell survival. Autophagy may make the last desperate efforts for survival in the dying cells.¹³⁹ Some studies have suggested that autophagy occurs ahead of apoptosis and necroptosis, and autophagy can promote or inhibit apoptosis/necroptosis. The ultimate goal of autophagy restraining apoptosis and necrosis may be to maintain cell survival. Once it cannot maintain survival, autophagy must promote cell death in a reasonable way to help maintain local homeostasis.^{140,141} From the study on GBMs toxicity, we can identify that the duty of autophagy is to degrade the intracellular proteins and abnormal cellular components, which may ultimately lead to cell death. However, there

is not enough evidence to prove that autophagic cell death occurs through autophagosome accumulation, and hence, more work is needed to distinguish the autophagic cell death induced by GBMs.

To clarify the mechanisms of GBM-induced PCD is beyond the scope of this review. However, the insights regarding GBM-induced PCD could be harnessed for more reliable assessments of the health effects of GBMs. The dose and time of occupational exposure of GBMs is huge, and hence, relative studies are not enough. Moreover, since GBMs can induce PCD in cancer cells, the application of GBMs in therapy will benefit to curing tumors/cancers combined with their powerful drug delivery function. However, we need to pay more attention to this study approach.

Abbreviations

BMDMs, bone marrow-derived macrophages; GBMs, graphene-based materials; GNPs, graphene nanoplatelets; GO, graphene oxide; GQDs, graphene quantum dots; MMP, mitochondrial membrane potential; MOMP, mitochondrial outer membrane permeabilization; PCD, programmed cell death; rGO, reduced graphene oxide.

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Disclosure

The authors report no conflicts of interest in this work.

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