

Invited Mini Review

Interplay between autophagy and programmed cell death in mammalian neural stem cells

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Mammalian neural stem cells (NSCs) are of particular interest because of their role in brain development and function. Recent findings suggest the intimate involvement of programmed cell death (PCD) in the turnover of NSCs. However, the underlying mechanisms of PCD are largely unknown. Although apoptosis is the best-defined form of PCD, accumulating evidence has revealed a wide spectrum of PCD encompassing apoptosis, autophagic cell death (ACD) and necrosis. This mini-review aims to illustrate a unique regulation of PCD in NSCs. The results of our recent studies on autophagic death of adult hippocampal neural stem (HCN) cells are also discussed. HCN cell death following insulin withdrawal clearly provides a reliable model that can be used to analyze the molecular mechanisms of ACD in the larger context of PCD. More research efforts are needed to increase our understanding of the molecular basis of NSC turnover under degenerating conditions, such as aging, stress and neurological diseases. Efforts aimed at protecting and harnessing endogenous NSCs will offer novel opportunities for the development of new therapeutic strategies for neuropathologies. [BMB Reports 2013; 46(8): 383-390]

INTRODUCTION

One of the greatest biological realizations in the history of modern science is that proper cell death has as an important role as cell survival and proliferation in human health and disease. Cell death is not an undesirable incident, but a strictly regulated series of events and constitutes essential part of normal life. Cell death is a cell-intrinsic suicide mechanism, tightly regulated by diverse cellular signals (1). This controlled nature of the cell death process through the initiation, execution and termination, has led to the neologism, "programmed cell death (PCD)" (2). PCD has evolved to achieve a delicate bal-

ance between cell survival and death in all metazoans. Aberrant regulation of this balance (too much or too little cell death) underlies a variety of human diseases, such as cancer, autoimmune diseases, and neurodegeneration, to name a few.

The exciting discovery that new neurons are generated from endogenous neural stem cells (NSCs) even in adult brains has sparked an immense interest in the research of NSC over the past decades. Multipotent NSCs are defined to possess the capabilities to give rise to all types of neural cells in the nervous system and to maintain the stem cell population by self-renewal (3). Due to these abilities to proliferate and differentiate into different neural lineages, NSCs are important in normal brain development and function, in the regulation of brain plasticity, and in the maintenance of tissue homeostasis in both embryonic and adult mammalian brains. Hence, the size of NSC pool needs to be finely regulated through cell death to ensure the appropriate development and function of the brain.

A great deal of interest in multipotent NSCs since their discovery in adult brains has boasted a number of publications. While most of them are focused on fate decision, differentiation, neurogenesis or cellular therapeutic application of NSCs, there has been relatively little effort to understand the basic biochemical mechanisms underlying the survival and death of NSCs. This mini-review discusses the mechanisms of PCD that regulate the turnover of NSCs. Although the PCD of NSCs shares many features with the PCD of neurons or other types of cells, it also has unique attributes pertaining to NSCs and presents challenges for comprehension of its molecular mechanisms. Increased understanding of this sophisticated and fascinating biological process will provide the groundwork for the utilization of NSCs in the treatment of a variety of neurological conditions.

CELL DEATH BY MANY PROGRAMS

Given the importance of PCD, it is not surprising that cell death is a very complicated process with multiple pathways. Currently, more than 11 pathways to cell death, 7 of which are observed in the central nervous system (CNS), have been identified (4). They can be classified into three types mainly according to morphological criteria along with other characteristics (Fig. 1) (5). Type I cell death or apoptosis is characterized by cell shrinkage, membrane blebbing, chromatin con-

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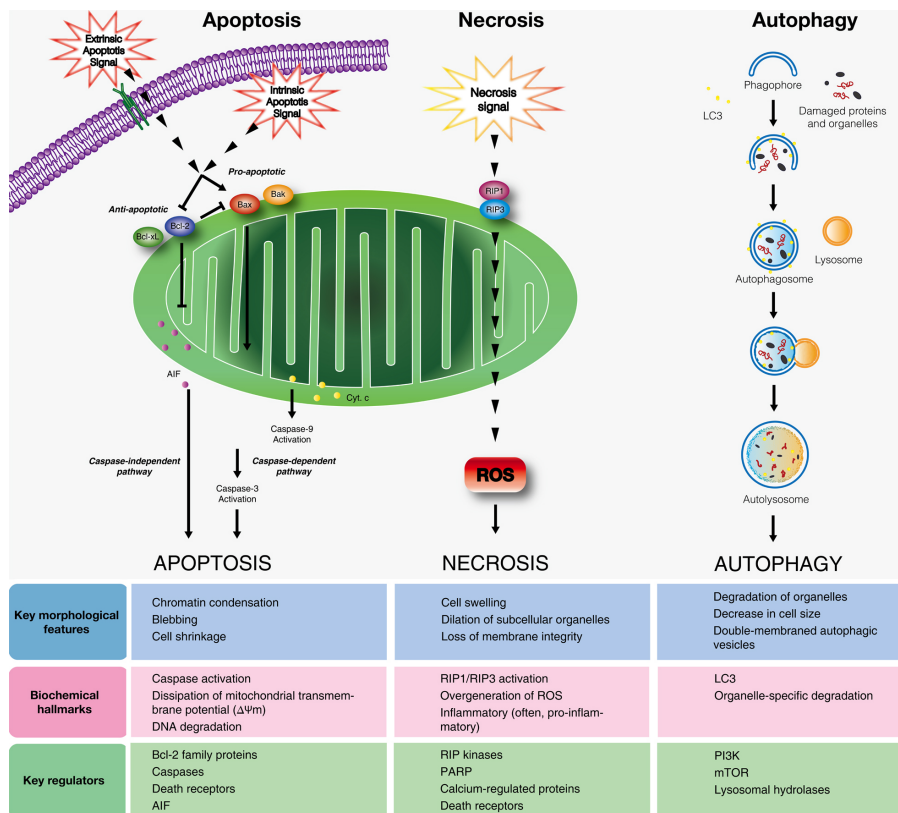


Fig. 1. A schematic illustration of each mode of PCD. Sequential induction of apoptotic (left), necrotic (middle), and autophagic (right) PCD in mammalian cells are shown with the major players of each pathway. Key morphological alterations, biochemical characteristics, and main regulators are listed in the table (bottom).

densation, dissipation of mitochondrial membrane potential, changes in plasma membrane lipid configurations, and nuclear DNA fragmentation. Apoptotic cells are packed into apoptotic bodies and cleared by neighboring phagocytic cells to prevent inflammation. Biochemically, caspase activation and nuclear DNA fragmentation are widely used to define apoptosis (6). Cells undergoing type II or autophagic cell death (ACD) display increased autophagic flux without the appearance of apoptotic markers. Autophagy is a cellular catabolic process by which cytosolic constituents including proteins and subcellular organelles are sequestered in double-membrane vesicles called autophagosomes and degraded by lysosomal hydrolases after fusion of autophagosomes with lysosomes (7). Long-lived proteins or damaged organelles are subject to degradation, and the resulting products are reused to provide metabolic intermediates. Therefore, autophagy helps to reduce cellular stress and maintain cellular homeostasis and cell viability. In contrast, the self-destructive role of autophagy has implicated autophagy mechanisms in cell death pathways (4, 8, 9). Lastly, type III cell death is necrotic cell death characterized by the swelling of several subcellular organelles and the rupture of the plasma membrane, which leads to the release of cellular contents and inflammation.

Apoptosis

Apoptosis is the most thoroughly investigated type of PCD. Kerr, Wyllie, and Currie observed the general occurrence of the natural cell death in a variety of tissues under normal physiological conditions and during development (10). Since then, there has been the explosive growth in our knowledge regarding the role of apoptosis in tissue development and homeostasis as well as in diseases (1, 11).

Mainly, apoptosis can occur by either extrinsic (death receptor-mediated) or intrinsic (mitochondria-mediated) biochemical pathways. Plasma membrane death receptors belong to the tumor necrosis factor (TNF) superfamily including TNF receptor 1, Fas, TNF-related apoptosis-inducing ligand receptors (TRAILR) and the p75 neurotrophin receptors (p75NTR) (reviewed in (12)). Upon binding of the extracellular ligands, death receptors recruit procaspase-8 and several adaptor molecules which have characteristic death domains and death effector domains. Procaspase-8 undergoes autoproteolytic activation and serves as the initiator caspase by triggering the activation of downstream executioner caspases, such as caspase-3 and -7 (reviewed in (13)). The intrinsic apoptosis pathway involves the mitochondria, which integrate and amplify cell death signals by release of pro-apoptotic molecules such as cytochrome c and apoptosis-inducing factor (AIF) (14). When located within the

mitochondria, cytochrome c is an essential component of the mitochondrial electron transport chain for generation of ATP. On the other hand, once released into the cytosol, it interacts with apoptotic protease activating factor-1, ATP and pro-caspase-9 to activate caspase-9 (15). Caspase-9 then activates the downstream executioner caspases. Activation of these downstream executioner proteases is the key biochemical marker for the apoptosis assay and is responsible for the major morphological and biochemical manifestation of apoptotic cells.

One distinct morphological change that occurs during apoptosis is cell shrinkage, which is associated with an increased efflux of K^+ and Cl^- ions through the ion channels in the membrane (16). The increased K^+ and Cl^- efflux reduces the intracellular K^+ concentration, facilitating the activation of caspases (16).

Although caspase activation is popularly used as a surrogate marker of apoptosis, cells can opt to undergo an alternative, caspase-independent cell death, such as AIF-dependent cell death (17). AIF is a mitochondrial flavoprotein, and like cytochrome c, is essential for normal mitochondrial physiology, since genetic ablation of AIF induces a deficiency in oxidative phosphorylation (18, 19). Conversely, once released into the cytosol, extramitochondrial AIF translocates to the nucleus and initiates caspase-independent chromatolysis and cell death (20). Thus, AIF represents another case of a single protein with dual roles in life and death of cells.

A recent study demonstrated differential regulation of neural precursor cells and NSCs by AIF in the hippocampus (21). This study used Harlequin mutant mice which express lower levels of AIF than wildtype mice. The investigators found that AIF down-regulation increased the survival of neural precursor cells, but not that of NSCs following cerebral hypoxia. Interestingly, another recent report showed that low oxygen-induced enhancement of survival of early embryonic (primitive) NSCs is mediated through the inhibition of AIF-dependent cell death, while low oxygen increased the survival of late embryonic and adult brain (definitive) NSCs through the inhibition of caspase-dependent cell death (22). These differential regulations of cell death in populations of proliferating neural precursor cells and NSCs, and also within subpopulations of NSCs strongly imply that PCD in NSCs is a delicately and tightly controlled event depending on the developmental stage.

p53 is a tumor suppressor protein that can initiate apoptosis. p53 can regulate the proliferation and survival of NSCs by controlling NSC self-renewal (23). p53 knockout mice displayed an increase in NSC proliferation and a decrease in apoptosis both *in vivo* and *in vitro*, the latter of which showed more pronounced phenotypes (23). It is now well-established that p53-mediated expression of several pro-apoptotic genes such as Bax plays a key role in cell death induction (24). A recent study demonstrated that oxidative stress-induced activation of p53 in NSCs is an upstream event of caspase-2 activation by Bax, suggesting that p53 is a critical regulator of the intrinsic mitochondria-mediated apoptotic pathway in NSCs (25).

Direct, clear evidence of the significant role of apoptotic cell death in NSCs was experimentally provided by knockout studies. Bax and Bak, the central pro-apoptotic Bcl-2 family proteins in the regulation of apoptosis, were revealed to regulate the size of the NSC pool (26). The Bax/Bak double-knockout mice displayed masses of NSCs in neurogenic niches, subgranular zone (SGZ) and subventricular zone (SVZ). This increased size of the NSC pool is solely the result of the reduction in cell deaths as proliferation is not affected. Both neural precursor cells and mature neurons derived from Bax/Bak mutant mice were resistant to various apoptotic stimuli, indicating that deletions of Bax and Bak can preclude apoptotic cell death. Due to the redundant and compensating role of each protein, single knockouts of Bax and Bak failed to have similar effects on the size of the NSC pool and susceptibility of cells to apoptosis.

Fas, a member of the TNF death receptor superfamily, is another well-known key molecule in regulating apoptosis (27, 28). A prior study found that the Fas-dependent cell death pathway is not operative in NSCs in spite of the intact mitochondrial pathway and caspase-dependent apoptotic machinery, suggesting the possibility of a different role of Fas in NSCs (29). In line with this, Fas ligand-induced activation of Fas receptor did not induce apoptosis in neural precursor cells, but rather increased cell viability and decreased apoptosis while not affecting proliferation (30). As a whole, death receptor-dependent apoptosis has been well characterized in immune cells, whereas their relevance in the brain, especially in NSCs, needs to be established in the future.

Autophagic cell death

The brain is specially protected against nutrient deficiency by a constant supply of nutrients from other organs even under starvation conditions, and consequently autophagy is not activated in response to nutrient starvation (31). However, this does not indicate the absence of autophagy in the brain; rather it demonstrates that constitutive activity of autophagy proceeds at a basal rate in the brain. Therefore, abnormal alteration in brain autophagy represents a critically dysregulated state.

Autophagy in a normal state plays a cytoprotective role by eliminating damaged organelles and proteins, but has also been shown to contribute to cell death if overwhelmed or induced excessively (32). Recent evidence of the presence of autophagic vacuoles in dying cells gave a rise to the term autophagic cell death (ACD). Despite the wide occurrence of autophagy in cells undergoing apoptosis or necrosis, possibly suggesting a mechanistic link between autophagy and PCD, the interrelation between autophagy and PCD appears to vary by cell type and stimulus (reviewed in (33)). ACD is an intriguing phenomenon because it leads a cell to its own destruction by literally "eating itself."

Precaution should be taken since the presence of an increased number of autophagic vacuoles in dying cells does not necessarily mean that autophagy has a primary role in cell death (34). Because the causal relationship between autophagy

and cell death has not been firmly established in many reports, “cell death through autophagy” (autophagy can be a mechanism to execute cell death and manipulation of autophagic flux should efficiently affect cell death rate) sometimes has not been distinguished from “cell death with autophagy” (autophagy may be an irrelevant epiphenomenon or a futile attempt of dying cells to survive without causal connection with cell death). This confusion has led some to argue against the existence of ACD (35, 36). However, despite these controversies, accumulating evidence supports the indispensable existence of ACD (reviewed in (37, 38)). Recently, Shen and Codogno have proposed a new set of criteria for ACD and listed several experimental situations where ACD meeting these criteria exists (reviewed in (38)). The proposed new set of criteria for ACD are as follows: i) cell death occurs without the involvement of apoptosis; ii) there is an increase of autophagic flux, as well as an increase of the autophagic markers, in the cells undergoing ACD; and iii) both pharmacological suppression and genetic suppression of autophagy are able to prevent cell death.

Two convincing models of mammalian ACD meeting the newly-proposed criteria listed above are from neuronal cells (39, 40). The most genuine model of ACD presently known is ACD in hippocampal neural stem (HCN) cells derived from adult rat (40). The mechanisms underlying cell death of NSCs in response to degenerating stressors remain largely unknown, although the tremendous interest has risen in exploring the NSC biology. One of the best understood signals of NSC pro-

liferation is growth factors. Growth factors play an important role in regulating the size of the NSC pool by directly stimulating proliferation or cell cycle kinetics or modulating cell death pathways. To study how a given growth factor affects the proliferation of NSCs and induces cell death following withdrawal, we investigated the cell death mode of HCN cells following insulin withdrawal. Insulin plays a key role in the hippocampal function and HCN cells are dependent on insulin signaling for proliferation (41). The insulin signaling enhances cell survival through the activation of PI3K and Akt, which inhibits GSK3 and disinhibits mTOR at normal state. Upon insulin withdrawal, HCN cells undergo ACD through the inactivation of mTOR (40) which has a leading role in triggering autophagy via inhibition of the PI3K/Akt, “the insulin signaling,” pathway (Fig. 2). Insulin withdrawal increased autophagy flux and cell death, which could be attenuated upon the genetic suppression of autophagy. On the other hand, the promotion of autophagy using the mTOR inhibitor *rapamycin* increased cell death. HCN cells have intact apoptotic machinery and undergo typical apoptosis, as shown by staurosporine-induced cell death. Nevertheless, insulin-deprived HCN cells did not exhibit apoptotic hallmarks, such as caspase-3 activation or nucleosomal DNA fragmentation, indicating the non-apoptotic nature of HCN cell death induced by insulin withdrawal. A correlation of cell death rate with the level of autophagic flux and the absence of apoptosis strongly suggests the primary role of autophagy in cell death. Hence, fulfilling

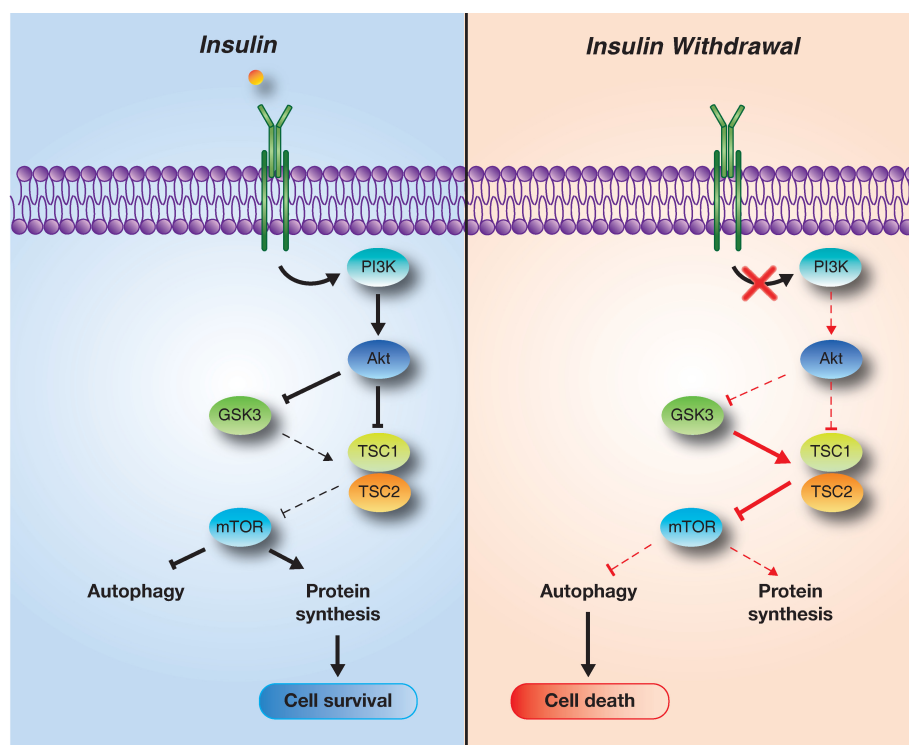


Fig. 2. A normal, physiological insulin signaling transduction (left) and an altered pathway following insulin withdrawal (right) in adult hippocampal neural stem cells. Insulin signaling enhances cell survival through activation of PI3K and Akt, which inhibit GSK3 and disinhibit mTOR at normal state. In contrast, insulin withdrawal leads to inactivation of PI3K and Akt, and subsequent disinhibition of GSK-3 and inhibition of mTOR, triggering autophagy induction.

the criteria listed above, insulin withdrawal-induced autophagic death in HCN cells represents a valid genuine model of mammalian ACD. Another model of ACD utilizes hypoxia/ischemia-induced neuronal cell death in both neonatal and adult mouse brains (39). Conditional knockout mice ($Atg7^{flox/flox}$; nestin-Cre) with specific deletion of *Atg7* in the CNS tissue showed nearly complete resistance to hypoxia/ischemia-induced cell death in the hippocampus, suggesting the possibility of ACD (39, 42). However, identifying this paradigm as a genuine model of ACD should not be concluded hastily since this hypoxia/ischemia-induced cell death in the hippocampus also accompanied the activation of caspase-3, as well as autophagic markers (42).

Necrosis

Traditionally considered as a passive form of cell death, necrosis is morphologically characterized by the breakdown of the plasma membrane and inflammation of the dying cell followed by the spillage of cellular contents and proinflammatory molecules. Necrotic cell death can be distinguished from apoptosis by the alteration in nuclear morphology while lacking the characteristics of apoptosis, namely chromatin condensation and DNA fragmentation. In contrast to the initial belief that necrosis occurs "by accident," several studies demonstrated evidence of "programmed" necrosis (reviewed in (43)). Programmed necrotic cell death is only observed under conditions in which apoptosis is blocked. Supporting evidence revealed that the apoptosis process suppresses necrosis through caspase-mediated cleavage and the inactivation of proteins required for programmed necrosis (44).

Crosstalk between autophagy and PCD

Redundancy (occurrence of several cell death modes in a single dying cell) and plasticity (ability to switch between different cell death modes) of PCD make it a daunting task to understand the complicated regulation mechanism of PCD and to find the critical nodules that control cell death. We can glean an inkling of intimate relationship between autophagy and PCD, especially apoptosis, through the action of common mediators, most notably Beclin 1 (45). Inhibition of apoptosis, in several cases, may activate autophagic or necrotic cell death (40, 44, 46, 47). Through the knockdown of *Atg7* and Beclin 1, Yu and others have revealed that PCD induced by inhibition of pro-apoptotic caspase-8 is mediated through *Atg7* and Beclin 1, illustrating that cells undergo ACD upon deactivation of apoptosis (46). Inhibition of caspase-3, the final executor of apoptosis, can also induce cell death with autophagic features (47). Inhibition of autophagy by caspase-3 through cleavage of Beclin 1 further supports the intimate relationship between autophagy and apoptosis in aspects of PCD (48). Furthermore, because Beclin 1 binding to anti-apoptotic Bcl-2 negatively regulates apoptosis, their interaction may coordinate the end result of PCD in dying cells. Interestingly, Bcl-2 can also function as an anti-autophagic protein as its interaction with Beclin 1 in-

hibits autophagy whereas phosphorylation of Bcl-2 dissociates the binding of Bcl-2 and Beclin 1 and induces autophagy (49).

UNIQUELY-REGULATED PCD IN NSCs

Resilience of NSCs to apoptosis

The tight control of NSC death can be assumed important from the fact that aberrant elimination of NSCs at any stage of brain development will have a dramatic effect on the final size of an NSC pool due to the exponential growth property of a single NSC.

Several experiments have shown distinct regulation of PCD in NSCs from other types of cells (50-52). For instance, one report showed different mechanisms of apoptotic cell death in adult NSCs and differentiated-neurons (50). In the study, the investigators discovered that the PCD of NSCs require both of pro-apoptotic Bax and Bak while the PCD process of post-mitotic neurons only requires Bax. Perhaps this stricter requirement in the induction of PCD in NSCs makes NSCs resistant to apoptosis. Since the deletion of Bax did not alter the proliferation of adult NSCs, Bax contributes solely to cell death and is likely the main effector of PCD in NSCs. Indeed, Bax was later found to be a major determinant of apoptosis in adult NSCs, acting through the modification of Ca^{2+} flux via caspase-3 activation (53). In addition, the intrinsic resilient nature of NSCs to death effectors may be responsible for the difference in PCD regulation. Supporting evidence includes higher levels of anti-apoptotic proteins Bcl-2 and Bcl-X_L in NSCs (54). Interestingly, the expression of p53 in NSCs is substantially higher than in the cells in other regions of the brain (23). A recent study provided strong evidence that ion flux may play a causative role in the difference between the cell death mechanisms in NSCs and neurons (51). The authors of the study focused on the apoptosis mediated through an opening of voltage-dependent anion channels in the plasma membrane (pI-VDAC). While hippocampal neurons undergoing apoptosis were accompanied by pI-VDAC openings, pI-VDAC was scarcely observed in NSCs, demonstrating different cell death mechanisms.

One factor confirming this unique regulation of PCD in NSCs can be found from neurogenic niches. In the CNS, the physiological concentrations of oxygen range from 0.55% in the midbrain to 8% in the pia (55). In particular, the physiological concentrations of oxygen in the NSC niches are at the lower end of the spectrum as the oxygen concentration in the SVZ is estimated to be 2.5 to 3% and the SGZ is located in the midbrain, the region with the lowest level of oxygen. The relatively hypoxic environment for NSCs may imply a possible regulatory role of oxygen in NSCs (56). Indeed, noticeably, two recent reports documented oxygen regulation of survival and death in NSCs (57, 58). One study revealed that hypoxia enhanced the survival and proliferation rates in hippocampal NSCs, while it decreased apoptosis (57). Another report found that exposure to hypoxia significantly reduced apoptotic cell

death in NSCs by promoting the activation of Wnt/ β -catenin signaling (58). The investigators further discovered that the modulation of hypoxia-inducible factor-1 α (HIF-1 α), a key mediator of hypoxic adaptation, mediates the Wnt/ β -catenin signaling and subsequent increase in cell survival. Moreover, the deletion of HIF-1 α led to impaired processes involved in the Wnt pathway, including NSC proliferation and differentiation. It is therefore conceivable that oxygen availability has a direct regulatory role in NSCs through its modulation of the Wnt pathway. In contrast, the loss of the Wnt/ β -catenin signaling resulted in increased cell death in neural progenitor cells but not in NSCs (52), whereas the upregulation of the signaling led to the loss of NSCs (59). These findings suggest that NSC and neural precursor cell populations may be differentially regulated through modulation of Wnt/ β -catenin signaling.

Programmed NSC death in neurogenesis

At the early stages of development, a massive PCD occurs in the brain, eliminating over half of the newly generated neuronal population following neurogenesis (60). During adult neurogenesis, PCD eliminates excess or unhealthy cells from neurogenic niches (61), perhaps as a regulatory mechanism to preserve the appropriate size of the NSC pool. By culling excessive cells, PCD contributes to a self-renewal mechanism in mammalian NSCs (60, 62). One underlying mechanism employs self-renewal regulation by Bmi1, a member of the polycomb group gene family, which enhances NSC proliferation and self-renewal ability (62). In the adult mammalian brain, neurogenesis constantly persists in two neurogenic regions, the SVZ and the dentate gyrus of the SGZ, where NSCs steadily produce new neurons throughout life (63). A new study revealed that neurogenic capability of mammalian brains wanes at old age due to an increased quiescence of NSCs, not due to the loss of NSCs (64). Most cells generated via neurogenesis undergo programmed apoptotic cell death, and the expression of apoptosis-related genes in NSCs are not altered with aging (64, 65), indicative of the intact existence of PCD in NSCs regardless of aging. Intriguingly, caspase-3, the executor of apoptosis, is intimately involved in NSC function as the activity of caspase-3 facilitates neurogenesis and is an indispensable requirement in NSC differentiation (66). This participation of caspase-3 is independent of the apoptotic events as the inhibition of caspase-3 has no effect on apoptosis (66).

Programmed NSC death in neurodegeneration

NSCs have garnered clinical promise in countering neurodegeneration due to their capacity to differentiate into appropriate cell types. NSC death is observed in several neurodegenerative conditions such as Alzheimer's disease (AD) and Parkinson's disease (PD) (8). One recent study particularly documented the susceptibility of NSCs to dopamine toxicity, a known key mechanism in the pathogenesis of PD (67). To examine the mechanism of selective dopamine toxicity-induced cell death, the investigators utilized immortalized human

NSCs and those transfected with the aromatic acid decarboxylase gene, the latter of which can be regarded as dopamine-secreting dopaminergic cells. Dopaminergic NSCs were found to be selectively susceptible to dopamine-induced apoptosis mediated through caspase activation (67). The capacity of NSC self-renewal and proliferation declines with aging and degenerative conditions, but there is very little known about how aging, stress and disease affect the function of endogenous NSCs. Understanding of the mechanisms by which neuropathological abnormalities predispose NSCs to cell death at the molecular level will have a huge impact on the utilization of endogenous NSCs for brain self-repair.

CONCLUSION

Utilization of endogenous NSCs in brain repair can be an ideal therapeutic approach as it may avoid many technical and ethical issues accompanying the transplantation of exogenous stem cells (68). However, it is important to point out that our knowledge about the effects of degenerating conditions on the function of endogenous NSCs is very limited. We can reason that a dynamic balance among self-renewal, maintenance of quiescent status, proliferation, differentiation and death may be under tight control by a variety of intrinsic and extrinsic signals and may be disturbed by degenerating stressors. A detailed examination of the roles of molecules that regulate this delicate balance will hold important implications in basic stem cell biology and cellular therapeutic application of stem cells for treatment of neurological disorders. The understanding of the various cell death programs on the molecular level will be a prerequisite for development of therapeutic interventions in diseases caused by dysregulated cell death. In this regard, HCN cells should prove to be an excellent model system for analyzing the biochemical mechanisms of ACD at a comparable level to apoptosis.

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