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REVIEW

Glioblastoma, hypoxia and autophagy: a survival-prone ‘ménage-à-trois’

Soha Jawhari¹, Marie-Hélène Ratinaud¹ and Mireille Verdier^{*,1}

Glioblastoma multiforme is the most common and the most aggressive primary brain tumor. It is characterized by a high degree of hypoxia and also by a remarkable resistance to therapy because of its adaptation capabilities that include autophagy. This degradation process allows the recycling of cellular components, leading to the formation of metabolic precursors and production of adenosine triphosphate. Hypoxia can induce autophagy through the activation of several autophagy-related proteins such as BNIP3, AMPK, REDD1, PML, and the unfolded protein response-related transcription factors ATF4 and CHOP. This review summarizes the most recent data about induction of autophagy under hypoxic condition and the role of autophagy in glioblastoma.

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Facts

- Glioblastoma multiforme (GBM) is the most aggressive brain tumor. Despite its high degree of hypoxia, it can survive and resist anticancer treatments. Thus, it is important to study the main GBM adaptive strategies.
- Autophagy is a catabolic process that can be induced by hypoxia. In most cancer cases, it leads to cell survival and resistance to anticancer treatments.
- The only study about hypoxia-induced autophagy in GBM highlighted its role in cell protection against stress and in the resistance to the antiangiogenic drug bevacizumab.
- Owing to its autophagy inhibition properties, chloroquine is currently used for its antitumoral effects in GBM. Indeed, phase III clinical trials have shown an increase in median survival for patients with GBM following surgery, chemotherapy and radiotherapy.

Open Questions

- Which signaling molecules induced by hypoxia are able to trigger autophagy?
- Why autophagy has a dual role in cancer: tumor suppression and tumor facilitation?

- Given the apparently contradictory effects of autophagy in the response of GBM to treatment (i.e., tumor cell invasiveness and senescence), how autophagy inhibition could be efficient in cancer therapy?

Gliomas originate from an uncontrolled proliferation of glial cells, and consist mainly of primary central nervous system tumors derived from astrocytes or oligodendrocytes. Several authors have successively tried to build a classification of gliomas and the scientific community currently recognizes the World Health Organization (WHO) classification¹ that has recently been updated.² This classification defines the tumor histological type according to the predominant cytological type; the tumor grade, from I to IV depends on the following criteria: increase in cell density, nuclear atypia, mitosis number, vascular hyperplasia and necrosis.³ Glioblastoma multiforme (GBM), a grade IV glioma,¹ is the most common and most aggressive malignant primary brain tumor⁴ whose cell type of origin has not yet established. GBM accounts for 60–70% of all glial tumors⁵ with an incidence of 3–4 cases per 100 000 individuals per year.⁶ Palliative treatment ensures 6–9 months as median survival, which is extended to 12 months after radiotherapy and 16 months after radiochemotherapy. Postoperative survival varies from 12 months (50%) to 24 months (20%) and reaches 36 months in 2% of

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Abbreviations: AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; ATF4, activating transcription factor 4; ATG, autophagy-related genes; Atg, autophagy-related proteins; ATP, adenosine triphosphate; Bcl-2, B-cell lymphoma 2; Bcl-x_L, Bcl-2 homolog X long protein; *BECN1*, gene encoding Beclin1; BNIP3, Bcl-2/adenovirus E1B 19 kDa interacting protein 3; Caspase, cysteinyl aspartate-specific proteinase; CHOP, C/EBP homologous protein; CoCl₂, cobalt chloride; CQ, chloroquine; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; FoxO3, forkhead box O3; GBM, glioblastoma multiforme; GLUT1,3, glucose transporter-1,3; GTP, guanosine triphosphate; HCQ, hydroxychloroquine; HIF1, hypoxia-inducible factor 1; IDH, isocitrate dehydrogenase; LC3, light chain 3; 3-MA, 3-methyladenine; mTOR, mammalian target of rapamycin; mTORC, mTOR complex; O₂, dioxygen; p16Ink4a, p16 cyclin-dependent kinase inhibitor 4a; PI3K, phosphatidylinositol 3 kinase; PML, promyelocytic leukemia; PTEN, phosphatase and tensin; REDD1, regulated in development and DNA damage responses 1; RHEB, Ras homolog enriched in brain; shRNA, small hairpin RNA; siRNA, small interfering RNA; *TP53*, gene encoding p53; TSC1/TSC2, tuberous sclerosis complex 1 and 2; ULK1, Unc-51-like autophagy-activating kinase 1; UPR, unfolded protein response; UVRAG, UV radiation resistance-associated gene protein; VEGF, vascular endothelial growth factor; WHO, World Health Organization

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cases. GBM occurs at all ages,⁷ but is more frequent between 45 and 70 years (70% of cases).⁸ It constitutes the second leading cause of cancer death in children after leukemia and the third one in adults. Death is usually due to cerebral edema, which causes an increase in intracranial pressure, and a reduced level of vigilance.⁹ GBM is a highly hypoxic tumor; deep and remote areas of the tumor suffer from a low dioxygen (O₂) partial pressure, which can drop down to 1%. Although one would expect that this condition should slow tumor growth, cancer cells eventually develop processes allowing them not only to survive hypoxia, but also to become more aggressive. Among these adaptive responses, autophagy, a catabolic process, leads in most cases to the survival of tumor cells. This survival pathway allows the degradation of different cell components with the production of energy (adenosine triphosphate (ATP)) and metabolic precursors further recycled by the cellular anabolism.

Glioblastoma: Molecular Alterations and Histology

GBM can appear 'de novo' and quickly evolve without any prior injury; it is then called primary GBM. It can also develop slowly (5–10 years) from a low-grade astrocytoma (grade II or III), and then is called secondary GBM.¹⁰ Primary GBM represent over 90% of these tumors. They develop most often in patients aged over 60 years and are characterized by a brief clinical history (about 1 year after diagnosis).¹⁰ Secondary GBM are rare (10% of them) and occur in younger patients (average age 45). They are characterized by a longer clinical history and have a better prognosis than primary GBM.¹⁰ These two types of GBM are morphologically similar but present different genetic alterations (Table 1). Indeed, primary GBM are essentially characterized by an amplification of *EGFR* (epidermal growth factor receptor) (36%), a deletion of *p16Ink4a* (p16 cyclin-dependent kinase inhibitor 4a) (31%) and a mutated *PTEN* (phosphatase and tensin homolog) (25%). In contrast, *TP53* (gene encoding p53) mutations are rare (30%).¹¹ On the opposite, secondary GBM are primarily characterized by *TP53* mutations (65%), amplification of *EGFR* is not so frequent (8%) as well as *p16INK4a* deletion (19%) and *PTEN* mutation (4%). Monosomy 10 is observed in almost 70% of GBM, whether primary or secondary.¹⁰ This monosomy can affect the whole chromosome 10 or only the long arm (loss of heterozygosity 10q) especially in primary GBM.¹² Genetic alterations have been recently discovered in

Table 1 Percentage of mutations of particular genes in primary and secondary glioblastomas

Mutated genes	Primary glioblastoma	Secondary glioblastoma	References
<i>EGFR</i>	36%	8%	11
<i>p16INK4a</i>	31%	19%	11
<i>PTEN</i>	25%	4%	11
<i>TP53</i>	30%	50%	11
<i>IDH1</i>	5%	80%	14–16

The difference in the occurrence of certain mutations between the two types of glioblastomas allowed its molecular classification. Whereas primary glioblastoma is mainly characterized by mutations in *EGFR*, *p16INK4a* and *PTEN*, secondary glioblastoma harbors *TP53* and *IDH1* mutations.

IDH genes, encoding isocitrate dehydrogenases 1 (*IDH1*) and 2 (*IDH2*).¹³ *IDH1* alterations are present in secondary GBM, but are rarely found in primary GBM; this difference allowed to discriminate between these two tumors types.^{14,15} Mutations in *IDH1/IDH2* are of real diagnostic value because they also allow the confirmation of a glial tumor, and the distinction between grade II gliomas and pilocytic astrocytomas (grade I).¹⁵ Indeed, these mutations have not been detected in any pilocytic astrocytoma, suggesting that these tumors proceed from another mechanism. Over 70% of low-grade gliomas present mutations in *IDH1* or *IDH2*.¹⁵ On the contrary, only 5% of primary GBM are affected and 80% of secondary GBM have a mutated *IDH1*.¹⁶ The last WHO classification version incorporates new entities to divide GBM into *IDH*-wild-type GBM (90% of cases that corresponds to primary GBM) and *IDH*-mutant GBM (10% of cases that corresponds to secondary GBM).²

As for histological characteristics, GBM consists of two distinct tumor areas:

- A very high cell density area associated with atypical nuclei and a high mitotic index. The tissue consists only of tumor cells with the presence of newly formed blood vessels (micro-angiogenesis).
- An area in which isolated cancer cells invade the parenchyma that remains morphologically and functionally intact and devoid of newly formed blood vessels.¹⁷ The isolated cancer cells reflect the infiltrating nature of GBM; they are characterized by atypical and elongated nuclei.³

Necrotic areas are found in GBM, which correspond to hypoxic regions surrounded by highly anaplastic cells.³

Hypoxia in GBM

Hypoxia means a low O₂ partial pressure (1 < pO₂ < 10 mm Hg). It therefore constitutes a limitation on the availability of O₂ in many diseases,¹⁸ such as brain and cardiovascular ischemia, diabetes and atherosclerosis. Hypoxia is also detected in solid cancers, particularly, in brain tumors such as GBM. In this type of tumors, hypoxic areas can be explained in different ways. First, the uncontrolled proliferation of cancer cells results in the formation of vessel-remote and highly hypoxic areas. Cancer cells react to this low partial pressure of O₂ by stimulating neovascularization (from pre-existing blood vessels). Indeed, hypoxic cells secrete VEGF (vascular endothelial growth factor). After binding to its receptor on the surface of endothelial cells, VEGF promotes the building of new vessels that help to provide the tumor cells with the required amount of O₂ and nutrients, thus ensuring their survival.¹⁹ This also leads to their dissemination in the body via the newly formed blood vessels rendered permeable by the VEGF signaling.²⁰ Nevertheless, this tumor vascular network can in turn contribute to the formation of hypoxic areas. In fact, neovascularization gives rise to abnormal, too small, occluded vessels, or capillaries or arteriovenous bifurcations (imperfect vascularization).²¹ The result is an insufficient supply of O₂, which maintains the hypoxic areas, and constitute a selection pressure that favors the rise of highly aggressive cells. Indeed, hypoxia-resistant cells trigger highly conserved signaling pathways to overcome the stress.

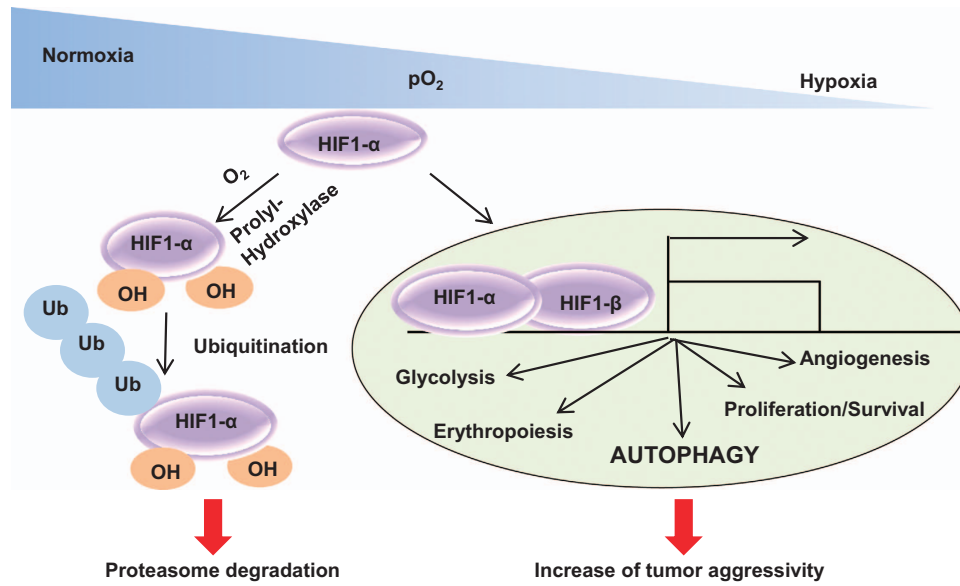


Figure 1 Hypoxia stabilizes HIF1 and promotes cell survival. In normoxia, HIF1- α is hydroxylated and then degraded by the proteasome. However in hypoxia, the absence of dioxygen leads to HIF1- α stabilization and complexation with HIF1- β . The HIF1 α /HIF1- β complex is transcriptionally active, and following the binding of various coactivators, can target specific genes including those involved in autophagy, survival, proliferation and angiogenesis

Not only can these cells adapt to low O_2 ,²² but they also resist anticancer treatments.²³

The response and the adaptation of cells to hypoxia are controlled by transcription factors activated by a low partial pressure of O_2 . The most important transcription factor in this homeostasis is the hypoxia-inducible factor 1 or HIF1 (Figure 1). It consists of a heterodimeric protein composed of one α and one β subunit, which present some sequence and structural domain homologies. These two subunits are differentially expressed.^{24,25} HIF1 β is constitutively expressed regardless of the availability of O_2 .²⁶ In contrast, HIF1 α has a short half-life ($t_{1/2} \sim 5$ min) and is highly regulated by O_2 .²⁷ In normoxia, HIF1 α is subjected to hydroxylation, which allows recruitment of the von Hippel–Lindau protein, polyubiquitination and further degradation by the proteasome.²⁸ However, in hypoxia, HIF1 α is stabilized and transferred from the cytosol to the nucleus, where it dimerizes with HIF1 β . The HIF1 α /HIF1- β assembly becomes transcriptionally active,²⁶ and several coactivators may join this complex to target and refine the transcription of specific genes, such as p300/CBP (CREB (cAMP response element-binding)-binding protein), TIF2 (transcription intermediary factor 2) and SRC-1 (steroid receptor coactivator-1).²⁹ It has also been reported that HIF1- α can be influenced by mutations in mitochondrial enzymes. Among these, IDH1 that is essentially mutated in secondary GBM,¹⁶ has been correlated with an increase in HIF1- α protein level and transcriptional activity in GBM cell lines.³⁰ However, this positive link has to be considered in relation with the tumor grade. Indeed, a recent study showed that the expression of HIF1A, as well as its downstream targets, is significantly reduced in grade II (low-grade diffuse) and III (anaplastic) gliomas harboring mutated IDH1/IDH2.³¹

At present, > 100 genes have been identified as targets of HIF1.³² When expressed, these genes allow the cells to cope

with hypoxia,³³ they promote angiogenesis (VEGF)³⁴ and cell survival (insulin-like growth factor-2),³⁵ they boost glucose metabolism (glucose transporter-1,3—GLUT1,3)³⁶ and facilitate tumor invasion (matrix metalloproteinases—MMPs).³⁷

Hypoxia may also induce an intracellular recycling mechanism, called autophagy, allowing cancer cells to resist the hypoxic stress.^{38,39}

Autophagy

Autophagy is a catabolic mechanism highly conserved during evolution. It is found in yeast, plants, worms, flies, mice and humans.⁴⁰ Autophagy is currently a focus of major scientific interest.^{41,42} Besides the proteasome, which mainly degrades short-lived proteins, autophagy eliminates, via lysosomes, altered organelles, long-lived and misfolded proteins. Autophagy occurs in response to nutrient starvation or oxidative stress, and leads to the formation of metabolic precursors (amino acids and fatty acids) and ATP, hence ensuring homeostasis and cell survival.⁴³ However, inadequate repairs or major stress can lead to cell death, named autophagic death.⁴⁴

Different types of autophagy are currently described: microautophagy, macroautophagy and chaperone-mediated autophagy.⁴⁵ Macroautophagy is the major recycling process of cytosolic components through the lysosomal pathway and is referred to as ‘autophagy’ in the review (Figure 2). It appears from isolated membranes (or phagophore) that rearrange dynamically. This results in a double-membrane structure called autophagosome,⁴⁶ which may include a portion of cytoplasm. The autophagosomal membrane probably originates from an infolding of the endoplasmic reticulum (ER) membrane.⁴⁷ At the end of the process, the autophagosome fuses with a lysosome to form the autophagolysosome where

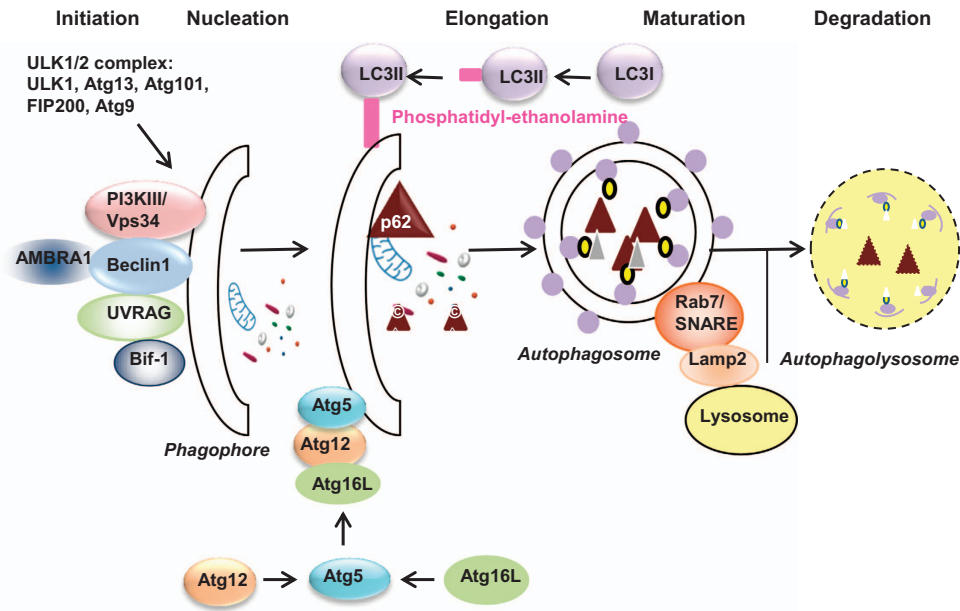


Figure 2 Autophagy process and molecular actors. Several external stimuli activate the ULK1/2 complex, which initiates isolation membrane near material to be removed. After this initiation step, the nucleation relies on the association of Beclin1 with class III phosphatidyl-inositol 3 kinase (PI3K III) to initiate phagophore formation. Other proteins like UVRAG, Bif-1 and AMBRA1 can also join this complex. The Atg5-Atg12-Atg16L complex and LC3 are two conjugation systems promoting the elongation of the phagophore to form the autophagosome, a vesicle containing the autophagic cargos. Cytosolic-free LC3, named LC3I, is transformed into LC3II, which consists of LC3 covalently bound to a phosphatidyl-ethanolamine molecule. LC3II is then anchored to the pre-autophagosomal membrane. After the binding of certain cytosolic cargos, the p62 protein leads them to the autophagosome, facilitating their degradation by autophagy. Thereafter, the autophagosome fuses with a lysosome to form the autophagolysosome, then the autophagic cargos are degraded by the lysosomal enzymes. Until its degradation, the p62 protein remains associated with the autophagosome

the sequestered material is degraded by the lysosomal acid hydrolases.^{48,49} The process provides metabolic precursors after their release into the cytosol by membrane permeases. The molecular machinery is orchestrated by the autophagy-related genes (*ATG*) family and autophagy-related proteins (*Atg*).⁵⁰ These genes and proteins have been first characterized in the yeast *Saccharomyces cerevisiae*.⁵¹ In mammals, many genes (>30) are involved in the genetic control of autophagy. Proteins are classified into three subgroups: the ATG1/ULK1 (Unc-51-like autophagy-activating kinase 1) complex and its regulators, the Beclin1/phosphatidyl-inositol-3 kinase class III complex, and the Atg5-Atg12 and LC3 (light chain 3) conjugation systems.

- The ATG1/ULK1 complex is also called the initiator complex of autophagy. It is regulated by the mTOR protein (mammalian target of rapamycin), a kinase forming two complexes, named mTORC (mTOR complex)1 and mTORC2. In nutrient-rich conditions, mTORC1 phosphorylates and inhibits the ATG1/ULK1 complex, thus preventing the initiation of autophagy.⁵² In nutritional deficiency conditions or after the addition of rapamycin (mTORC1 inhibitor), mTORC1 dissociates from the complex, decreasing the phosphorylation level of the ATG1/ULK1 complex that becomes active⁵³ and initiates autophagy.⁵⁴ The mTORC2 complex (rapamycin insensitive) can also inhibit autophagy via activation of Akt. The latter activates mTORC1, but also phosphorylates and inhibits FoxO3

(forkhead box O3; reviewed in Hay⁵⁵) that normally activates the transcription of important autophagy genes, such as *BNIP3* (B-cell lymphoma 2 (Bcl-2)/adenovirus E1B 19 kDa interacting protein 3) and *LC3* genes.⁵⁶

- Formation of the Beclin1/class III phosphatidyl-inositide 3 kinase (PI3K) complex ensures autophagosome nucleation.⁴⁷ Beclin1 activation requires its release from Bcl-2 and/or Bcl-x_L (Bcl-2 homolog X long protein).⁵⁷ Indeed, Beclin1 is inactive when it is bound to these proteins.⁵⁸ Other proteins such as UVRAG (UV radiation resistance-associated gene protein),⁵⁹ Bif-1⁶⁰ and AMBRA1 (activating molecule in Beclin1-regulated autophagy)⁶¹ could interact with Beclin1 to participate in autophagy processing and/or regulation.
- The ubiquitin-like Atg5-Atg12 and LC3 conjugation systems are necessary for autophagosome elongation.⁶² After its conjugation in the cytosol, the Atg5-Atg12-Atg16L complex transiently binds to the pre-autophagosomal membrane⁶² and facilitates the recruitment of the MAP-LC3 (microtubule-associated protein 1 (MAP1) LC3) protein or LC3.⁶³ The free form of the latter (LC3I) is transformed into the lipidated form LC3II by the covalent binding of a molecule of phosphatidyl ethanolamine.^{64,65}

This modification is needed to anchor LC3 to the pre-autophagosomal membrane.^{64,66} LC3II remains bound to the autophagosome until its degradation (Figure 2).⁶⁷ This conversion of LC3I to LC3II is a key marker of autophagy,

more particularly when the autophagic flux is inhibited. Indeed, a functional autophagy must lead to the degradation of cargos, which can be evaluated in the presence of lysosomal inhibitors such as chloroquine (CQ) or bafilomycin A1. These inhibitors induce autophagosome and LC3II accumulation in the case of an efficient and functional autophagy. However, evidencing autophagy induction requires that autophagic flux is inhibited, otherwise LC3II would be rapidly degraded and recycled.⁴¹

The p53 protein is another important actor of the regulation of autophagy; according to its localization, it can activate or inhibit autophagy. The cytosolic p53 inhibits the formation of autophagic vacuoles⁶⁸ by interaction with FIP200 (FAK (focal adhesion kinase) family kinase-interacting protein of 200 kDa), whereas nuclear p53 acts as a transcriptional factor of genes implied in autophagy regulation. For example, PTEN, which is an important negative regulator of the Akt/mTOR pathway.⁶⁹ The p53-dependent expression of the lysosomal protein DRAM (damage-regulated autophagy modulator) activates macroautophagy.^{70–72} Moreover, we have already shown that the efficiency of the signaling is dependent on its genetic profile (wild type or mutated). Indeed, p53 does not always induce autophagy as we evidenced in neuroblastoma cell lines in response to cobalt chloride (CoCl₂, a hypoxia-mimicking agent). In the wild-type *TP53* neuroblastoma cell line (SHSY5Y), CoCl₂ induced an apoptotic signaling involving p53, characterized by a decrease in mitochondrial membrane potential, and activation of cysteinyl aspartate-specific proteinases (caspases) 9 and 3. In the *TP53*-mutated neuroblastoma cell line or in the wild-type cells with invalidated *TP53* expression (by small hairpin RNA (shRNA)), CoCl₂ induced an autophagic signaling. It precedes cell death, occurring later in the absence of caspase activation, but with the release of the mitochondrial apoptosis-inducing factor, favoring an autophagic cell death.⁷³ Another regulator example is AMPK (adenosine monophosphate (AMP)-activated protein kinase), whose role will be detailed below in the section 'Autophagy and Hypoxia', as this protein is being activated under hypoxia condition.⁷⁴

Autophagy and GBM

Today, the data on the inhibition and/or the development of a tumor via autophagy seem contradictory. Autophagy presents a dual function in cancer.^{75,76} In the early stages of tumorigenesis, it would limit cell proliferation, DNA damage and therefore tumor progression. When the tumor size increases, its blood supply is restricted, and autophagy may promote the survival of tumor cells in the nutrient-deficient and hypoxic tumor regions.⁷⁷ Furthermore, autophagy, as already evoked, may lead to either cell survival or death.

In particular, it has been shown that *BECN1*, the gene encoding Beclin1, is deleted in 75%, 50% and 40% in ovarian, breast and prostate cancers, respectively.⁷⁸ The mutation of a single allele of *BECN1* increases the incidence of tumors.⁷⁹ Deletion of *ATG7* has also been reported in several cancers.^{80,81} In metabolic stress conditions, autophagy inhibition realized by inactivation of *BECN1* or *ATG5* increases DNA damage and chromosomal instabilities.⁸² Thus, autophagy seems to prevent damage to DNA and limit the occurrence of cancers. Finally, the tumor suppressor protein,

PTEN, induces an autophagic signaling, whereas it is inhibited by oncogenes.⁸³ However, the autophagy ability to recycle the intracellular contents protects tumor cells from adverse environmental conditions. Thus, this signaling appears as an adaptive mechanism in poorly vascularized solid tumors. It ensures independence in nutrients and energy necessary for the survival of tumor cells in hypoxic regions.⁸⁴ Indeed, as already described above, hypoxia causes the stabilization of HIF1 α . This activates the transcription of several target genes such as *VEGF*, leading to tumor neovascularization³⁴ and *GLUT1-3*, ensuring the transport of glucose.³⁶

These contradictory aspects are also observed in the case of GBM. Indeed, autophagy can positively or negatively regulate both GBM invasion and resistance to therapy, the two hallmarks of GBM aggressiveness.⁸⁵ Concerning invasion and cell motility, whereas Catalano *et al.*⁸⁶ reported an impairment of migration and invasion of GBM cells after autophagy induction, Palumbo *et al.*⁸⁷ noticed this impairment after autophagy inhibition, especially when EGFR was coinhibited, thus highlighting the importance of growth factor signaling.

When considering therapy resistance, the interplay between classical chemotherapy and autophagy regulation seems to be complex. For example, it has been recently shown that curcubitacin I, a potent anticancer molecule, induced an autophagy process in GBM cells and in a xenograft model, leading to cell survival and tumor protection.⁸⁸ However, itraconazole was shown to inhibit the development of GBM through an autophagic signaling induction, whereas the blockage of autophagy significantly reverses the anti-proliferative effects of this molecule, suggesting an antitumor role of autophagy in response to itraconazole.⁸⁹ Temozolomide (TMZ) (Temodal, MERCK, Kirkland, Quebec, Canada) is the most used drug to treat GBM; it induces autophagy in GBM cell lines and provides them with resistance to the treatment. As early as 2004, Kanzawa *et al.*⁹⁰ have reported an induction of survival autophagy in GBM cell lines, suppressing the antitumor effect of TMZ. The use of 3-MA (3-methyladenine, class III PI3K inhibitor) or bafilomycin A1 restored TMZ cytotoxicity, suggesting that autophagy inhibition, combined to TMZ in GBM treatment, could enhance the cytotoxicity of this chemotherapeutic agent.⁹¹ This twin treatment is currently the most promising strategy to limit tumor development.^{92–97} Indeed, CQ and its analog hydroxychloroquine (HCQ), which are the only autophagy flux inhibitors⁹⁸ approved by the US Food and Drug Administration (FDA),⁹⁹ are in a phase III clinical trial in adjuvant settings, following surgery, chemotherapy and radiotherapy. CQ-treated patients benefit from a clearly better median survival, compared with placebo-treated patients.¹⁰⁰ Despite the promising effect of CQ,¹⁰⁰ a recent phase I/II clinical trial combining HCQ with TMZ and radiation therapy, in patients with newly diagnosed GBM showed that the maximum tolerated dose of HCQ was unable to consistently inhibit autophagy and that dose-limiting toxicity prevented the possibility of moving to higher doses of HCQ.¹⁰¹ Such a study highlights the toxic side effect of this molecule, used in combination with classical chemotherapeutic agents.¹⁰² As a result, we are waiting for further development of novel autophagy inhibitors with less cytotoxicity.

In a contradictory way, another approach to GBM treatment could consist in targeting the autophagy inhibitor mTOR.^{42,103} Indeed, dysregulation of the PI3K/Akt/mTOR signaling pathway is common in human cancers, inducing uncontrolled cell proliferation, which can be targeted by rapamycin analogs. However, even if in GBM, the presence of autophagy inducers such as RAD001 (mTOR inhibitor) allowed TMZ to induce an autophagic death signaling,¹⁰⁴ only few cancers responded to such treatment. It appears that mTOR inhibitors should be associated with other growth factor pathway inhibitors^{103,105} or with autophagy inhibitors, considering they can also induce a survival autophagy. Thus, such twin treatment could counteract cell survival pathways, and at the same time, sensitize tumor cells to death. Furthermore, it has been shown that inhibition of mTOR could boost temozolomide-induced senescence, whereas autophagy inhibition triggered apoptosis and decreased senescence.¹⁰⁶ Nevertheless, the link between autophagy and senescence needs to be clarified. Indeed, in a particular genetically engineered GBM mouse model in which autophagy was disrupted, gliomagenesis or GBM formation was significantly reduced or completely inhibited.¹⁰⁷ *In vitro* studies showed an intact cellular growth, but a real senescence induction essentially characterized by activation of retinoblastoma 1 (RB1/p105) and an increase in CDKN1B/p27 level.^{107–110} Thus, the induction of senescence, consecutively to autophagy dysregulation, needs to be investigated more thoroughly to constitute a hopeful therapy able to reduce GBM aggressiveness.

Finally, targeting autophagic process could also interfere with antiangiogenic therapy, frequently used in cancer. For example, the treatment of GBM cell lines with CQ in combination with 3-MA, ATG7 and BECN1 small interfering RNA (siRNA), blocked the autophagy induced by ZD6474, a small molecule that blocks the VEGF receptor (VEGFR) activity, thereby sensitizing GBM cells to ZD6474-induced apoptosis.¹¹¹ Indeed, GBM are characterized by their high capacity of angiogenesis¹¹² that is important for tumor growth, making antiangiogenic molecules crucial for GBM treatment.¹¹³ However, owing to their tumor devascularization capacity, these molecules cause increased intratumoral hypoxia, which in turn activates autophagy and finally help tumor cells to resist treatment.¹¹⁴ Thus, the antitumor effects of antiangiogenic agents seem to be enhanced when they are combined with autophagy inhibitors.¹¹⁵

Autophagy and Hypoxia

Several studies have reported a link between hypoxia and the induction of autophagy as summarized in Figure 3. In fact, the hypoxia factor HIF1 activates the transcription of the *BNIP3* gene,¹¹⁶ encoding the BNIP3 protein, which can induce autophagy. Indeed, BNIP3 displaces Beclin1 from Bcl-2 or Bcl-x_L by competing for the same binding site; free Beclin1 then induces autophagy.⁵⁷ BNIP3 can also activate autophagy by inhibiting the RHEB (Ras homolog enriched in brain) protein, whose activity is required for mTORC1 activation.¹¹⁷

In addition, the energy depletion caused by hypoxia increases the intracellular AMP/ATP ratio, which stimulates the monophosphate-activated protein kinase or AMPK, an energy-sensing switch.¹¹⁸ AMPK indirectly inhibits mTOR by

phosphorylation and activation of TSC2 (tuberous sclerosis complex 2) and Raptor, inhibiting the activity of mTORC1 and activating autophagy.¹¹⁹ Indeed, the TSC1–TSC2 complex contains a GTP (guanosine triphosphate)ase-activating protein domain, which stimulates the GTPase activity of RHEB and thereby preventing it to activate mTORC1.¹²⁰ In addition, AMPK regulates autophagy by phosphorylating ULK1^{121–123} and thus inducing mTOR-independent autophagy. AMPK also promotes the accumulation of FoxO3 in the nucleus and the expression of its autophagy-associated target genes (*LC3* and *BNIP3*).¹²⁴ Moreover, AMPK has been shown to be an important regulator of the HIF1 activity and of the expression of its target genes in prostate cancer cells cultured in the presence of CoCl₂.¹²⁵ Not only is the TSC1–TSC2 complex maintained in its active form (phosphorylation of TSC1) by AMPK, but it is also activated by the REDD1 (regulated in development and DNA damage responses 1) protein, itself activated under hypoxic condition; then autophagy is induced by preventing TSC2 phosphorylation and sequestration by the 14-3-3 protein.¹²⁶ An interesting study on GBM cell lines showed that hypoxia induced autophagy.¹¹⁵ This signaling was preceded by a stabilization of HIF1 α and an increase in phospho-AMPK (active AMPK). The extinction of one of these proteins by siRNA significantly reduced the autophagic process. Hypoxia-induced autophagy was also dependent on BNIP3, whose expression increases under hypoxic condition in GBM cell lines and in xenograft-derived cells. Inhibition of autophagy with CQ induced caspase-dependent apoptotic death, without affecting BNIP3 expression. Moreover, this hypoxia-induced autophagy promoted the tumor resistance to antiangiogenic therapy. Indeed, when GBM xenografts were treated with bevacizumab alone, an increase in BNIP3 expression and hypoxia-associated growth were recorded. Addition of the autophagy inhibitor CQ blocked this process. *In vivo*, ATG7 extinction in athymic mice by shRNA, also disrupted tumor growth especially when combined with bevacizumab treatment.¹¹⁵ Another hypoxia-activated protein, the tumor suppressor protein PML (promyelocytic leukemia) activates autophagy by binding mTOR, thereby preventing its interaction with RHEB, causing its deactivation and its nuclear accumulation.^{127,128}

Furthermore, under hypoxic conditions, the protein trafficking (secretion or incorporation in the plasma membrane) is defected because O₂ is required for protein maturation.¹²⁹ Hypoxia initiates the unfolded protein response or UPR, which can in turn induce autophagy through ATF4 (activating transcription factor 4) activation.¹³⁰ Indeed, ATF4, stabilized in hypoxia, is able to upregulate the LC3B protein, which is essential for the autophagic process. The inhibition of ATF4-activated autophagy significantly increases apoptosis and reduces cell viability in several hypoxic cancer cells.¹³⁰ Another study carried out on other cancer cells, including U373 MG, a GBM cell line, the hypoxia-induced UPR activated PERK (protein kinase RNA-like ER kinase) in the ER. The latter activates ATF4 and another transcription factor, the C/EBP (CCAAT/enhancer-binding protein) homologous protein or CHOP, which increases the expression of the *LC3B* and *ATG5* genes, thus inducing autophagy. Exposure to CQ sensitized cancer cells to hypoxia and xenografted tumors to irradiation.¹³¹

Conclusion

The impact of autophagy on GBM cell fate remains ambiguous. Autophagy could protect cancer cells from drastic environmental conditions such as hypoxia, and from chemo

and radiotherapy, but can also inhibit the cell growth and induce senescence, depending on the context. Autophagy induced by chemotherapeutic agents may either promote or inhibit cell survival, depending on the drug used. As for the hypoxia-induced autophagy in this tumor, it has been shown

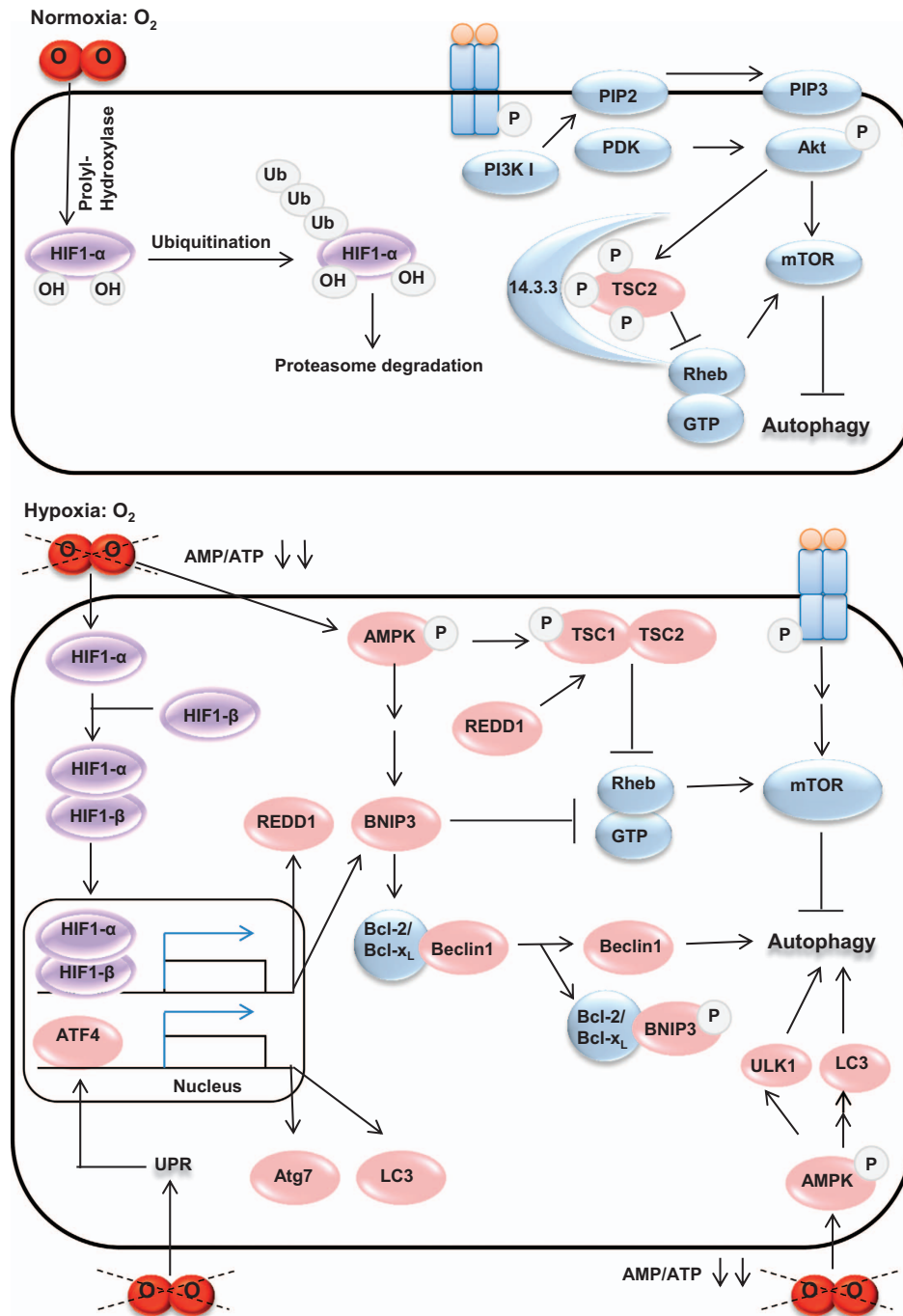


Figure 3 Different actors involved in the induction of autophagy by hypoxia. In normoxia, HIF1- α is hydroxylated and degraded by proteasome. As the presence of dioxygen is favorable to cellular proliferation, receptor tyrosine kinases that promote proliferation are activated, leading to mTOR activation and autophagy inhibition. However, in hypoxia, HIF1- α is stabilized and it associates with HIF1- β to form HIF1 that activates the transcription of several genes whose encoded proteins activate autophagy. For example, REDD1 activates the mTOR inhibitors, thus removing the autophagy inhibition. BNIP3 can also activate autophagy by decreasing the RHEB/GTP level. Moreover, BNIP3 is a HIF1 target, and it competes with Beclin1 for binding to Bcl-2 or Bcl-x_L, triggering the Beclin1 release and the induction of autophagy. BNIP3 can also be induced by AMPK that is activated in response to hypoxia. AMPK can also activate ULK1 and LC3, thus triggering autophagy. In hypoxic conditions, the unfolded protein response (UPR) activates the transcription factor, ATF4, which in turn activates Atg7 and LC3, two proteins involved in the autophagosome elongation

that autophagy was able to protect cancer cells against hypoxia, but also could enhance their resistance to antiangiogenic therapy.¹¹⁵ Furthermore, recent clinical studies have been conducted with CQ, which inhibits autophagy, or with mTOR inhibitors, which activates this process; both kinds of therapies resulted in beneficial effects on GBM evolution.^{42,102} These facts highlight the importance of autophagy modulation as a potent anti-GBM strategy that could improve the efficiency of chemotherapy.¹¹⁵ Thus, more studies are needed to elucidate the exact role of autophagy in GBM and to delineate the associated therapy.

Conflict of Interest

The authors declare no conflict of interest.

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