Autophagy in Osteosarcoma

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Abstract

Osteosarcoma (OS) remains a difficult disease to treat. The standard chemotherapy regimen has not improved survival for the past three decades. Resistance to chemotherapy remains a challenge and constitutes a major concern to clinical investigators. Autophagy has been recognized as a survival mechanism implicated in resistance to chemotherapy. We previously demonstrated chemotherapy to induce autophagy in OS. However, whether induction of autophagy will lead to survival or death has been the focus of many laboratories. Autophagy is a very context-dependent process, and no specific biomarker has been identified to define whether the process will lead to survival or death. In the present chapter, we present some of the mechanisms involved in the process of autophagy and summarize some of the most recent work related to autophagy in OS and the challenges encountered with the use of old and new autophagy inhibitors.

Keywords

Autophagy · Osteosarcoma · Chemotherapy · Survival · Death

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Introduction

The term autophagy derives from the Greek meaning "eating of self." It is a catabolic process by which cells self-degrade their own constituents to maintain homeostasis and allow regular turnover of cell components [1]. In mammals, three types of autophagy have been described: macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy involves bulk degradation of cytosol and organelles, microautophagy engulfs only parts of the cytosol or organelles, and chaperone-mediated autophagy involves the degradation of specific cytosolic proteins [2]. In this chapter, we focus on macroautophagy (hereafter referred as autophagy), the most studied autophagy type.

Under stressful conditions such as hypoxia, starvation, and cytotoxicity, autophagy allows the recycling of cellular components to be used as a source of energy. Autophagy is implicated in various different biological functions. Not only it plays a role in cell survival but it is also implicated in metabolism and development.

Autophagy has been shown to play an important role in many diseases such as neurodegenerative diseases where defects in autophagy can result in neurodegeneration [3]. It is also associated with aging [4] and the development of autoimmune diseases [5, 6], metabolic disorders [7], and cancer. Deregulation of autophagy has been described in many cancers such as glioblastoma,

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melanoma, lymphoma, and other solid tumors [1, 8]. In cancer, autophagy plays a role at different levels of cancer progression [1]and it is not associated with a specific trigger.

Autophagy could promote cell survival by protecting malignant cells from unfavorable conditions but could also serve as a tumor suppressor by impairing malignant transformation and promoting malignant cell death through programmed cell death (PCD) type II [1, 9]. This dual role of autophagy has been demonstrated in many cancers including osteosarcoma (OS). Therefore, targeting autophagy has been the focus of many studies [3, 10–12].

The process of autophagy involves more than 30 autophagy-related genes (Atg) and includes several steps. As shown in Fig. 11.1, the autophagy process starts when a stressful signal (1) activates the Atg1 complex, comprised of Atg1, Atg13, Atg17, Atg29, and Atg31, which leads to the formation of a flat membrane cistern, the phagophore, via activation of the vesicle trafficking complex formed by vesicle-mediated vacuolar protein sorting 34 (Vps34), a phosphatidyl inositol 3 kinase (PI3K), and one of the first characterized autophagy proteins, Beclin1. Interaction of these complexes and other factors help to recluse proteins and lipids necessary for the autophagosome formation (2). Completion of the autophagosome formation happens during elongation, the next step in the autophagy process (3). This step is regulated by two ubiquitin-like systems: the first system involves the formation of the Atg12, Atg5, and Atg16 complex, which is mediated by the E1-like enzyme Atg7. The second system regulates the conjugation of the microtubule-associated protein 1 light chain 3 (LC3-I/Atg8) with phosphatidylethanolamine (PE). LC3 is first synthesized as an unprocessed form, proLC3, and subsequently converted to a proteolytically processed form, LC3-I. LC3-I is cleaved by the protease Atg4, modified into the PE-conjugated form, LC3-II, and translocated from the cytoplasm to the autophagosome membrane. LC3 is the only known marker of the autophagosome (4). It also acts in cargo recognition by directly interacting with sequestosome 1 (SQSTM1/p62) via a complex formed between the cargo and SQSTM1 also bound to the autophagosome membrane. At this stage, the lysosome fuses to the autophagosome, forming the autolysosome (5). As a final step, proteins are degraded in the autolysosome and amino acids are released into the cytoplasm. These final products can be used for protein synthesis or can be oxidized by the mitochondria electron transport chain to produce adenosine triphosphate (ATP) to use as source of energy for cell survival. All

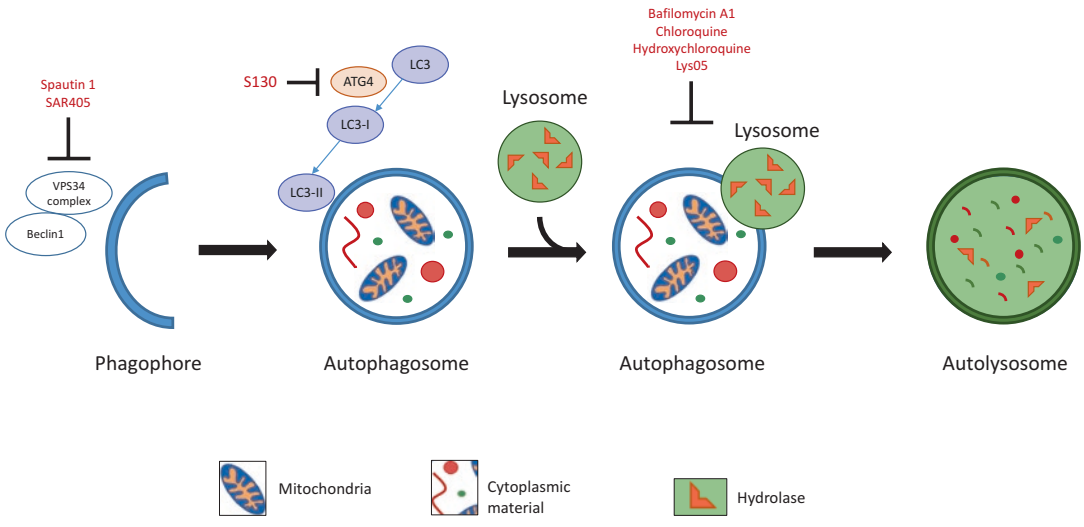

Fig. 11.1 The process of autophagy

proteins involved in the phagophore and autophagosome formation are released into the cytosol for reuse [13].

Regulation of Autophagy in Osteosarcoma

Autophagy is regulated through different mechanisms. The most studied mechanism involves the phosphoinositide 3 kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway. In fact, the nutrient sensor PI3K is upstream of the mammalian target of rapamycin (mTOR) kinase, which negatively regulates autophagy. During normal nutrient conditions, the PI3K/AKT/mTOR pathway is activated, leading to inhibition of autophagy [14, 15]. However, during periods of nutrient deprivation, the PI3K/AKT/mTOR pathway is inhibited leading to autophagy induction [16].

Another important mechanism that regulates autophagy and tumorigenesis involves Beclin-1. Beclin-1 is part of a multiprotein complex formed by Vps34/class III PI3K. This complex initiates the formation of the phagophore. The interaction of Beclin-1 with Vps34 is modulated by anti-apoptotic molecules such as Bcl-2 and Bcl-xL. Under normal nutrient conditions, Beclin-1 is bound to Bcl-2 or Bcl-xL inhibiting autophagy. During starvation or stressful conditions, Beclin-1 is disrupted from Bcl-2/Bcl-xL through phosphorylation of the binding domain. The Beclin-1 complex can also be disrupted by other mechanisms that involve binding of the complex to the DAMP molecule or high-mobility group box 1 (HMGB1). The end result is induction of autophagy [17, 18].

In OS, these and other mechanisms are involved in autophagy regulation. Activation of the PI3K/AKT/mTOR signaling pathway has been demonstrated to inhibit autophagy in OS. The use of rapamycin, an mTOR inhibitor, induced autophagy and increased cell death in MG63 human osteosarcoma cells. Combination therapy rapamycin and cisplatin further enhanced cisplatin-induced cytotoxicity and stimulated autophagy [19]. Using a similar approach, arsenic trioxide in combination with radiation therapy induced autophagy and increased cytotoxicity in the HOS human OS cells through a mechanism that involves inhibition of the PI3K/AKT/mTOR signaling pathway [20]. Tumor-suppressing STF cDNA 3 (TSSC3) inhibition of the Src-mediated PI3K/AKT/mTOR signaling pathway induces autophagy and increases cytotoxicity of mineralized tissue-forming (MTF) osteoblasts and SaOS2 human OS cells [21]. Similarly, treatment of LM7, CCH-OS-D, and K7M3 metastatic OS cell lines with gemcitabine induces autophagy through a decrease in AKT and mTOR phosphorylation [12]. Furthermore, induction of Beclin-1 has also been shown to induce autophagy in OS. Panobinostat, a histone deacetylase inhibitor, suppresses Bcl-2 in SaOS2, U2-OS, and MG63 human OS cells and increases Beclin-1 expression leading to induction of autophagy and increased cytotoxicity [22]. Targeting MiR-100 inhibited mTOR, increased Beclin-1, and induced both autophagy and apoptosis in OS [23]. HMGB1-mediated autophagy induction leads to chemotherapy resistance in MG63, U2-OS, and SaOS2 human OS cells. Inhibition of both HMGB1 and autophagy led to increased drug sensitivity [24, 25]. A more recent study linked COP9 signalosome subunit 3 (COPS3), a protein-coding gene, to autophagy regulation and metastasis formation in OS [26].

Further, epigenetic alterations have been shown to play an important role in regulating the process of autophagy [13, 27–29]. Epigenetics involves the various mechanisms that allow for certain genes to be turned on and off under specific circumstances. Stable alterations in gene expression are essential for the development and differentiation of cells. Any abnormality in the regulatory process could lead to tumorigenesis. Several epigenetic mechanisms have been described that modulate gene expression such as DNA methylation, histone modifications, and nucleosome remodeling [29]. These mechanisms play important roles in gene transcription and regulation of gene expression. Several transcription factors that influence the process of autophagy have been identified. P53 and forkhead box O3 (FOXO3) were the first two transcription

factors shown to induce autophagy [27]. Transcription factor EB (TFEB) is considered a key transcriptional regulator of autophagy as it activates the whole autophagy-lysosome pathway [30]. Under normal nutrient conditions, Zink Finger With KRAB and SCAN Domain 3 (ZKSCAN3) and Fork head transcription factor long isoform (FOXK) act as transcriptional repressors by inhibiting autophagy gene expression. The previous deleted reference should go as a number reference. Further, certain histone modifications can alter autophagy regulation [31] by having a direct effect on certain autophagy genes or by interacting with intermediates of the signal transduction pathway for autophagy. H4K16 acetylation and H3K9 dimethylation regulate core autophagy genes, whereas H3K27 trimethylation activates mTORC1 signaling leading to autophagy inhibition [27]. Bromodomain protein 4 (BRD4), a histone reader, links histone modifications to autophagy gene expression. BRD4 functions to inhibit autophagic activity under nutrient repletion status and knocking down BRD4 sustains autophagy during starvation status [27]. In pancreatic ductal adenocarcinoma, where autophagy has been described as a major resistance mechanisms to standard therapy, BRD4 was shown to be increased after gemcitabine treatment and contributed to drug resistance. Silencing BRD4 impaired cell viability and proliferation [32]. There is so far very limited knowledge on how epigenetic modifications can regulate autophagy in OS. Previous studies demonstrated that histone deacetylase inhibitors (HDACI) such as Trichostatin A inhibits the mTOR signaling pathway, enhances FOXO1 transcriptional activity, induces autophagy, and decreases cell death in human U2OS OS cells. Further inhibition of autophagy caused a marked enhancement of Trichostatin A-induced cell death in U2OS cells, suggesting potential efficacy of this combination for the treatment of OS [33].

Lastly, noncoding RNAs such as the small nucleolar RNA Host Gene 6 (SNHG6) can act as an oncogene in OS and induce autophagy through the regulation of Unc51-like autophagy activating kinase 1 (ULK1), a member of the preinitiation autophagy complex. Induction of autophagy through this mechanism decreases OS cell viability. Further silencing of the noncoding RNA SNHG6 inhibits OS cell growth and invasion [34].

In summary, various mechanisms are involved in the regulation of autophagy. None of them are specific to OS or any other disease process. Autophagy is a very context-dependent process, and its outcome might potentially be determined by the status and regulatory mechanisms triggered at the time the autophagy process is induced.

Autophagy and Tumorigenesis

Cell Survival Versus Cell Death

Autophagy exerts a dual role in tumorigenesis. It can either promote cell survival or cell death [35–37].

Autophagic cell death or programmed cell death (PCD) type II is described as a cell death mechanism that occurs in the presence of lysosomes. It differs from apoptosis (PCD type I) and necrosis (PCD type III) in that it lacks the chromatin condensation seen in apoptosis and swelling of the organelles seen in necrosis [38]. Autophagic cell death is caspase independent and can occur in the absence of proapoptotic proteins such as Bcl-2-associated X (Bax) and Bcl-2 homologous antagonist killer (Bak). In addition, during autophagic cell death, there is an increase in C-Jun N-terminal kinase (JNK), an essential cell death signaling molecule. However, insufficient JNK causes uncontrolled cell growth [39]. Certain chemotherapeutic agents can induce autophagy and lead to autophagic cell death. An example is obatoclax, a Bcl2 inhibitor, in acute lymphoblastic leukemia and Quinacrine in ovarian cancer [40, 41].

Alternatively, inability of cells to undergo autophagic cell death has been associated with tumorigenesis [42]. To this end, autophagy induction in cancer cells can also support tumor growth through various different mechanisms. It can induce cell survival during nutrient and oxygen

shortage, promote chemotherapy resistance, and prevent apoptosis [43]. For example, in pancreatic cancer, under specific conditions, inhibition of autophagy causes tumor regression suggesting a potential contribution of autophagy in pancreatic tumor growth [44]. Indeed, induction of autophagy in pancreatic stellate cells within the tumor microenvironment was found to promote tumor growth [45]. Similarly, the role of autophagy in tumor growth has also been attributed to the tumor host autophagy status. In the face of an autophagy-competent host, autophagy leads to tumor growth. This is highlighted in a recent paper by Katheder et al. where dormant tumor cells from autophagy-deficient *Drosophila* reactivated tumor growth when implanted in an autophagy-competent host, emphasizing the potential role of host autophagy in tumorigenesis [46]. This duality has been described in various tumors including OS.

Dual Role of Autophagy in Osteosarcoma

As previously stated, autophagy has been described as a mechanism that is context dependent. Previous studies developed in our laboratory demonstrated autophagy to have a dual role in OS. Different OS cell lines and treatments were used. In the murine OS cell lines K7M3 and DLM8, we demonstrated that treatment of these cells with camptothecin(CPT) induced autophagy. However, inhibition of autophagy led to decrease CPT-induced cell death in DLM8 and increase in CPT-induced cell death in K7M3 OS cells [47]. Treatment of the two human OS cell lines, LM7 and CCH-OS-D, with the nucleoside analog, gemcitabine(GCB), also led to induction of autophagy. However, inhibition of autophagy in the LM7 cells caused increased cell death, whereas inhibition of autophagy in the CCH-OS-D cells led to an increase in cell survival confirming the dual effect of chemotherapy-induced autophagy in OS [12]. This duality is not species specific as the effect was seen in mouse (K7M3 and DLM8) and human (LM7 and CCH-OS-D) cells. It is not specific to any particular chemotherapy agent as different chemotherapeutic agents (CPT,GCB) with different mechanism of actions led to the same dual effect. There is still very limited understanding of the underlying mechanisms that define these responses. Many factors and pathways have been described as responsible for either increase in cell survival or death. However, this effect has so far been attributed to the specific context where autophagy takes place. Santiago O'Farril et al. are the first ones to describe the potential for a small heat shock protein to define autophagy outcome in OS. We describe this effect in the next section of the chapter.

Heat Shock Proteins and Autophagy

Heat shock proteins (HSPs) are a class of functionally related proteins whose expression is increased when cells are exposed to elevated temperatures and other types of stress. HSPs protect cells from stress-associated injury, are overexpressed in many malignancies, and are implicated in tumor cell proliferation, differentiation, invasion, and metastases. Santiago O'Farril et al. identified phosphorylated Hsp27(pHSP27) as a potential biomarker to determine whether autophagy induction will lead to survival or death in OS. Induction of pHSP27 following drug exposure with GCB correlated with the role of autophagy in drug sensitivity. Blocking autophagy in OS cells whose pHsp27 was increased following drug exposure with GCB resulted in enhanced drug sensitivity. However, blocking autophagy in OS cells where pHsp27 was decreased resulted in reduced cell sensitivity. These findings are the first to identify the potential of this heat shock protein to act as a biomarker to define the specific conditions where inhibition of autophagy will provide benefit [12]. Additionally, further studies demonstrated that positive expression of HSP27 and negative expression of LC3B in OS correlated with the worst 10-year overall survival, whereas negative HSP27 expression and positive LC3B expression had the best 10-year overall survival which suggested HSP27 as a negative prognostic marker in OS [48]. Other HSPs have

also been described to play a role in autophagy induction. HSP90AA1 which belongs to the HSP90 family of HSP is upregulated in OS. It promotes autophagy and inhibits apoptosis leading to chemotherapy resistance [10]. The specific link between autophagy and heat shock proteins in OS is yet to be identified. However, these findings warrant future investigations on the potential role of HSPs in the modulation of autophagy in OS.

Autophagy Inhibition: From Drug Development to Challenges into Clinical Translation

Autophagy is a universal process present in every cell. Under physiologic conditions, autophagy is required to maintain tissue homeostasis. However, it can also contribute to the development and progression of certain diseases such as cancer. The development of autophagy inhibitors has become a challenge. Several drugs targeting autophagy have been described in the literature. Some compounds target the initial steps of the autophagy process, whereas others target autophagy at a later stage altering lysosomal functions [49]. Table 11.1 describes the different drugs that serve as autophagy inhibitors.

Early-stage inhibitors include pan-PI3K inhibitors such as 3-methyladenine (3-MA), which was first described in 1982 as a drug that acts to inhibit autophagy [50]. It was not until later when 3-MA was found to target both, class I PI3K and Vps34. 3-MA is nonspecific and poorly soluble which limits its potency [49]. More novel pan-PI3K inhibitors have been developed, but to date, none of those compounds have been shown to potently inhibit autophagy [49]. Another family of early stage inhibitors targets the Vps34 complex, a key structure in the autophagy process. Spautin-1 promotes the degradation of Vps34 complexes and causes cancer cell death under nutrient-deprived conditions. A preclinical study has shown synergistic effect of spautin-1 in combination with imatinib in the treatment of chronic myeloid leukemia [51]. SAR405, a pyrimidinone compound, was recently identified as a potent and selective catalytic inhibitor of Vps34, and it was shown to trigger an antiproliferative effect in renal cell carcinoma when combined with everolimus, an mTOR inhibitor, [49].

Late-stage autophagy inhibitors block the degradation of the autophagosome contents by the lysosomes. Bafilomycin A1 is a vacuolar-type H^+ ATPase inhibitor which blocks lysosomal proton transport thus inhibiting autophagic flux [49]. Inhibition of autophagy using bafilomycin A1 helped overcome chemotherapy resistance in gastric cancer cells [52].

There are also the so-called lysosomotropic agents used for the treatment of malaria. These include chloroquine (CQ) and hydroxychloroquine (HCQ). These agents disrupt lysosomal acidification and inhibit autolysosome formation. The major side effect of CQ is retinal toxicity. The addition of a hydroxyl group in HCQ ameliorates this effect by decreasing its ability to cross the blood-retinal barrier. CQ and HCQ are well tolerated. Efficacy of these agents in various

Table 11.1 Autophagy inhibitors

Compound	Target	Characteristics
3-Methyladenine	pan-PI3K inhibitors	Nonspecific, poorly soluble
Spautin-1	Vps34 inhibitors	Degrades VPS34 complexes and causes cancer cell death
SAR405	Vps34 inhibitors	Potent and selective catalytic inhibitor of Vps34
Bafilomycin A1	Blocks degradation of autophagosome contents	Inhibits autophagy flux to overcome chemotherapy resistance
Chloroquine	Inhibits autolysosome formation	Major side effect: retinal toxicity
Hydroxychloroquine	Inhibits autolysosome formation	Less retinal toxicity than CQ
Lys05	Inhibits autolysosome formation	More potent than CQ and HCQ
S130	ATG4 inhibitor	Potent inhibitor, causes cancer cells death in vitro

preclinical studies warranted their use in clinical trials. There are currently 31 active clinical trials using HCQ in combination with other drugs for the treatment of various malignancies. Temozolamide in combination with HCQ for the treatment of solid tumors and melanomas was tested in a Phase I clinical trial and demonstrated to be well tolerated with no associated toxicities [53]. However, an additional phase I/II trial that tested the same combination but with the addition of radiotherapy was used in patients with glioblastoma multiforme. The results demonstrated no improvement in survival at the chosen dose and severe myelosuppression at higher doses [54]. In vitro preclinical studies demonstrated effectiveness of the combination therapy HCQ + GCB in OS. A more recent Phase I study to explore the safety and tolerability of HCQ in combination with GCB and docetaxel (NCT03598595) in patients with recurrent or refractory OS was initiated and is ongoing.

Uncertainties remain with the use of chloroquine derivatives. A recent meta-analysis combined data from seven clinical trials using CQ and HCQ in combination with chemotherapy or radiation therapy and demonstrated that autophagy inhibitor-based therapy had a better treatment response than chemotherapy or radiation alone [55]. However, whether CQ/HCQ effectively inhibits autophagy in human tumors remains controversial. Potency at the tolerable doses remains suboptimal. Other derivatives are under development. Lys05, a bivalent analog of HCQ, has a tenfold greater potency than HCQ and demonstrated a better antitumor activity in preclinical models of glioblastoma, colon cancer, and melanoma [56, 57].

Additional autophagy inhibitors are under development. S130 targets the inhibition of ATG4. S130 tested in vitro demonstrated arrested growth of colorectal cancer cells and induced cell death [58].

In summary, here we describe the various autophagy inhibitors available and address their mechanism of action. Identification of an effective autophagy inhibitor remains a challenge. Further studies are needed to elucidate the best and more suitable autophagy inhibitor to use and in addition identify specific biomarkers of response.

Summary

The role of autophagy in OS remains unclear. Here, we describe autophagy as a mechanism that can either lead to survival or death in OS. We also point to some of the mechanisms implicated in the regulation of autophagy as it relates to OS. No one mechanism defines the outcome of autophagy in this disease. Furthermore, there isn't a well-identified biomarker to define the autophagy fate in OS whether it is induced by chemotherapy or other kinds of stress. We describe the potential for HSP27 to determine whether induction of autophagy will lead to survival or death, summarized the different autophagy inhibitors available, and point to the remaining challenges on the selection of one specific inhibitor. Better understanding of the mechanisms involved in the induction of autophagy in OS is necessary to define its role and select the most appropriate and effective agent to specifically target the process.

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