Editorial

A cytological link between radioresistance and autophagy

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Spontaneous and acquired resistance to radiation is one of the major dilemmas in cancer therapy. Numerous methods, e.g., neoadjuvant and adjuvant concurrent chemoradiotherapy (1), have been implemented to increase radio-sensitivity of tumor cells, and such regimens have markedly improved patient's survivals. The effect, however, is limited.

Radio-resistance has been associated with the decreased local oxygen level (hypoxia), nutrient deprivation, the reduced intracellular reactive oxygen species (ROS), and the elevated DNA repair-associated mechanism. Therefore, eliminating these factors might increase therapeutic efficacy. Interestingly, hypoxia and nutrient deprivation not only reduce production of ROS, an indication of abridged mitochondrial function, but also induce autophagy, a process that removes damaged mitochondria. An elegant study by Chaachouay *et al*. confirmed that hypoxia, in particular at the clinical oxygen concentration, induced autophagy, and increased cell survival following irradiation (2). However, they found that the cytoprotection effect of hypoxia might not be triggered by an AMP-activated protein kinase (AMPK), but fortuitously through an anti-apoptosis pathway.

In fact, autophagy has, in recent years, emerged as a major event of cellular homeostasis when cells are under micro-environmental stress (3). In some cases, autophagy, as an extension of innate immune host defense mechanism, is commenced to capture the escaped intracellular pathogens (4).

Biochemically, the canonical autophagy is initiated when unc-51-like autophagy activating kinase 1 (ULK1) [yeast homolog is autophagy-related gene 1 (Atg1)] is activated, which then phosphorylates membrane-bound ATG9 to attract the binding of ATG18 and to tether ATG9 vesicles (phagophore-related vesicles). Moreover, ULK1 phosphorylates ATG6 (Beclin-1) to mediate the formation of class III phosphoinositide-3-kinase (PIK3C3) complex, a lipid kinase which contains six stoichiometric subunits, vacuolar protein sorting 34 (VPS34), p150 (yeast Vps15), Atg14-like protein (ATG14L), UV radiation resistance-associated gene (UVRAG) protein, Rubicon and ATG6 (5). Beclin-1, VPS34 and p150 form an active core component, while ATG14L, UVRAG and Rubicon constitute a regulatory module. The activated ULK1 also assembles with Atg13 and Atg17 to form a vesicle nucleation center that in turn interacts with coiled-coil at N-terminus of ATG9 on the phagophore (6). This process is called PIK3C3 (VPS34)/Beclin 1-related pathway, which includes two routes of ATG conjugation: (I) formation of ATG12-ATG5-ATG16 triplex: first, ATG12 interacts with ubiquitin-like-conjugating enzyme E1 protein (ATG7), then switches to ATG10 (E2), and then conjugates to ATG5. Subsequently, ATG12-ATG5 binds ATG16 to form an ATG12-ATG5-ATG16 triplex aggregate, which is an E3, catalyzing conjugation of the edited ATG8 [microtubule-associated protein 1A/1B-light chain 3 (LC3-I)] to a phosphatidylethanolamine (PE), on pre-autophagosome membrane. The ATG8-PE conjugate is named as LC3-II (3); (II) lipidation of ATG8 [mammalian homologs are LC3, GABAA receptor-associated protein (GABARAP), and Golgi-associated ATPase enhancer (GATE-16) (7)]: following removal of the C-terminal 22 amino acids by a cysteine protease ATG4, the edited ATG8 then binds ATG7 (E1), and then switches to ATG3 (E2). Successively, ATG8, which covalently binds to ATG3, is conjugated to the head group of phosphatidylethanolamine (PE) by

E3 ligase (i.e., the ATG5-ATG12-ATG16 triplex) (8). The ATG8-PE conjugate (also known as LC3-II) is located on the membrane of autophagosome.

During this process, Rab7 is recruited to the autophagosome and interacts with lysosome-associated membrane protein 1 (Lamp1) and Lamp2 to facilitate the fusion of lysosome with the conjoining autophagosome. Formation of autophagolysosome enables disassembly of the aged or damaged organelles and digestion of the sequestered proteins (9). The increased pool of amino acids in turns activates kinase activity of mammalian target of rapamycin (mTOR) complex 1 (mTORC1) to phosphorylate zinc-finger transcription factor, ZKSCAN3, which is translocated to the nucleus to repress expression of autophagy-related genes, and to restore the cellular homeostasis (10).

In addition to ATG cascade, three ER membrane-integrated proteins, double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol regulating enzyme 1 (IRE1) in unfolded protein response (UPR) are also important in regulating autophagy. During UPR, Calcineurin is provoked to dephosphorylate transcription factor EB (TFEB) and transcription factor E3 (TFE3). TFEB and TFE3 are then translocated to the nucleus to induce expression of autophagy-related genes, while in the cytoplasm, formation of autophagosome ensues (10). As noted above that autophagy is an adjustment process of cellular homeostasis when cells encounter severe physiological and/or pathological anomaly, in particular during microbial infection, which concurrently induces ER stress, mitochondrial aberrations and damage to the DNA, the pivotal determinant of radiation sensitivity (11).

Like UVRAG, yeast *rad23* mutant is sensitive to UV light (12). Rad23 [mammalian homologs, human homologue of Rad23A (hHR23A) and hHR23B] is hence considered correlates with DNA repair. All rad23 proteins contain an N-terminal ubiquitin-like domain (UBL), and two highly conserved ubiquitin-associated domains (UBA), indicating that rad23 may interact with xeroderma pigmentosum complementation group C (XPC) protein to facilitate nucleotide excision repair (NER). Interestingly, silencing hHR23A reduces nucleolar levels of dynamin-related protein 1 (DRP1) when cells are exposed to hypoxic condition, suggesting that hHR23A is vital for nuclear import of DRP1, and DRP1 is essential for nucleolar protection (13). Chiang *et al*. showed that DRP1, the AAA domain containing 3A (ATAD3A) and mitofusin 2 (Mfn2), are crucial for intracellular material transport to mitochondria (14). Interestingly, knockdown of ATAD3A (ATAD3A^{kd}) reduced expression of DNA repair-related genes, e.g., ataxia-telangiectasia-mutated (ATM) kinase, γ-H2AX, and Nijmegen breakage syndrome 1 (NBS1). Nuclear levels of these proteins are also reduced in ATAD3A^{kd} cells, suggesting that ATAD3A also plays an important role in nuclear import, which, surprisingly, is karyopherin-independent (11). Fascinatingly, serum starvation increased expression of ATAD3A (15), and hypoxia elevated DRP1, further suggesting an intracellular feedback mechanism to provide the required, but insufficiently supplied materials.

It is worth noting that there is another type of cytoplasmic vesicles, which are closely associated with phagocytosis and endocytosis. This type of cytoplasmic vesicles is frequently observed when a receptor is binding by the corresponding ligand, which then activates class I phosphatidylinositol 3-kinase (PI3K) and the downstream protein kinase B (PKB)/AKT to initiate endocytosis. PI3K also maintain kinase activity of mTOR to prevent autophagy. However, serum starvation or nutrient deprivation stimulates activities of tuberous sclerosis complex (TSC) 1/2, which then inhibits mTOR function and initiates autophagy (16). The activated PI3K, on the other hand, recruits Rab5 and Rab7 on lysosome (17), and ATG9 on phagosome to prepare the fusion of two organelles to form a phagolysosome. This type of cytoplasmic vesicle formation is thus called ligand-dependent (or external factor-induced phagocytosis). Membranes of this type of vacuoles measure approximately 9–10 nm and are coated with signal transduction-associated proteins, such as clathrin (18). The vacuoles that originated intracellularly, on the other hand, are generated by autophagy (1,11,13-15) or intracytoplasmic phagocytosis reacting to the escaped microbes (e.g., *Listeria monocytogenes*, in innate immunity) (4). Membranes of these vacuoles measure approximately 6–7 nm and are coated with LC3-II (3).

Taken together, these data reveal that there are at least three major pathways, which are related to initiation of autophagy: ligand-dependent (phagocytosis and endocytosis), ligand-independent (autophagy), and obstruction of intracellular material transport passage. In their report, Chaachouay *et al*. showed that BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) and hypoxia-inducible factor 1 alpha (HIF-1α), which were highly expressed, were still phosphorylated in AMPK^{kd} cells when cells were exposed to moderate or severe

hypoxic conditions. Especially, the BNIP3 (NP_004043.3), a member of pro-apoptotic Bcl-2 homology 3 (BH3)-only protein with potential transmembrane domain (a.a. 226-248) at the C-terminus, was highly phosphorylated when cells were exposed to hypoxia. In epithelial and mouse embryonic fibroblast cell lines, such phenomena were mostly evident.

An elegant study by Liu and Frazier recently demonstrated that phosphorylation in the C-terminus of BNIP3 prevented mitochondrial impairment, probably due to the electrostatic repulsion of charged BNIP3 to insert into mitochondrial outer membrane (19). Their findings provided strong support for Chaachouay's observations, and a reasonable explanation for the anti-apoptotic effect of hypoxia on resistance to radio- and probably to chemo-therapy. It should be kept in mind though that in actual fast growing tumor nest, due to the features of neo-vasculature which contains only capillaries with sluggish blood flow, supply of oxygen and nutrients as well as growth factors and hormones must be simultaneously running below sufficient. In this case, the normally phosphorylated ATG5, which are maintained by the growth arrest and DNA damage 45 beta (Gadd45β)—MAPK/ERK kinase kinase 4 (MEKK4)—p38 MAP kinase (Gadd45β-MEKK4-p38) pathway (20), would be dephosphorylated because of the lack of growth factors. The sequel would be activation of ATG12 and ATG5 conjugation, shortage of crude material for protein synthesis and impediment of intracellular material transport to mitochondria, the symptomatic signs of autophagic initiation. Prolonged insufficiency would provoke cell death, resulting from either apoptosis or necrosis, as shown by the necrotic center of tumor nests.

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Footnote

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