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Tumor cells can evade dependence on autophagy through adaptation

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ABSTRACT

The autophagy-lysosome and the proteasome constitute the two major intracellular degradation systems. Suppression of the proteasome promotes autophagy for compensation and simultaneous inhibition of autophagy can selectively increase apoptosis in transformed cells, but not in untransformed or normal cells. Transformed cells are thus more dependent on autophagy for survival. However, it is unclear whether long-term autophagy inhibition/insufficiency would affect such dependency. To address this question, we transformed wild-type and autophagy-deficient cells lacking a key autophagy-related gene Atg5 with activated Ras. We found that such transformation did not make the autophagy-deficient tumor cells more susceptible to proteasome inhibitors than the wild type tumor cells, although the transformed cells were in general more sensitive to proteasome inhibition. We then compared the effect of acute versus constitutive knock-down of a key autophagy initiating molecule, Beclin 1, in an already transformed cancer cell line. In a wild-type U251 glioblastoma cell line (autophagy intact), increased sensitivity to proteasome inhibition was induced immediately after the knock-down of Beclin 1 expression with a specific siRNA (acute autophagy deficiency). On the other hand, when the tumor cell line was selected over a long period to achieve constitutive knock-down of Beclin 1, its sensitivity to proteasome inhibitors was no higher than that of the wild-type tumor cells. These results suggest that long-term autophagy deficiency either before or after oncogenic transformation can render the tumor cell survival independent of the autophagic activity, and the response to chemotherapy is no longer affected by the manipulation of the autophagy status.

1. Introduction

The ubiquitin–proteasome system and macroautophagy (referred to as autophagy hereafter) are two major cellular mechanisms for protein degradation. The proteasome mainly degrades short-lived regulatory proteins, while autophagy mostly targets long-lived functional proteins [1]. The malfunction of either pathway will affect cellular functions. Proteasome inhibition is cytotoxic in many cancer cells [2], and proteasome inhibitors, such as bortezomib (Velcade®), have been developed for treatment of refractory and relapsed multiple myeloma. They are also effective in suppression of other types of tumors such as leukemia [3].

The role of autophagy in tumor development is multi-faceted [4]. During the early stage of oncogenesis, autophagy can eliminate abnormal organelles such as the damaged mitochondria [5], thus helping to maintain the cellular homeostasis. However, in established tumors, the autophagy machinery can provide extra nutrients to meet the metabolic needs of the malignant cells. Thus autophagy has both pro-tumor and anti-tumor functions. As a specific metabolic pathway, autophagy is now considered as a new key regulator of cancer [6], and can serve as an effective therapeutic target. Considering the differential effects of autophagy and proteasome inhibition in tumor suppression, the combined targeting of the two pathways can be a potent strategy in cancer treatment.

Our previous studies have shown that compared to non-transformed cells, cancer cells rely more on autophagy to survive under adverse conditions [7]. When autophagy is suppressed, HCT116 colon cancer cells or DU145 prostate cancer cells undergo a significantly higher level of apoptosis following exposure to starvation

Abbreviations: MEF, murine embryonic fibroblasts; Atg5, autophagy-related protein 5; LC3, microtubule-associated protein 1 light chain 3; PE, phosphatidylethanolamine; PI3KC III, phosphoinositide 3-kinase complex III; PBS, phosphate buffered saline; RIPA buffer, radioimmunoprecipitation buffer; DMEM, Dulbecco's modified Eagle medium; PVDF, polyvinylidene difluoride; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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or proteasome inhibitors than the normal cells. In addition, a direct comparison between two immortalized isogenic ovarian cell lines, one was further transformed with Ras but the other was not, found that only the transformed line became more susceptible to proteasome inhibition in the state of autophagy suppression. No significant changes in death susceptibility were observed in non-transformed cells under the same treatment. The transformed line also responded more strongly to general autophagy stimulation. These studies thus suggest that autophagy is a much more crucial mechanism for transformed cells to survive, which has developed a dependency on this function.

In this article, we reported our studies on the issue of whether autophagy is equally important to cancer cells under the same condition of proteasome inhibition if they had lost autophagy activity early on. We would like to understand whether cancer cells can develop an adaptation to the low autophagy functionality.

2. Materials and methods

2.1. Antibodies and chemicals

The following antibodies were used: anti–β-actin (Sigma), anti–caspase-3 (Cell Signaling), anti-Beclin 1 (Santa Cruz), horseradish peroxidase–labeled secondary antibodies (Jackson Immuno Research Lab), rabbit anti-LC3II polyclonal antibody [7]. All chemicals were from Sigma, Invitrogen, or Calbiochem.

2.2. Cell lines and cell culture

Wild-type and Atg5-deficient MEFs were cultured in DMEM with standard supplements and were immortalized using SV40 large T [8]. Oncogenic H-Ras was introduced into MEFs using pBabe-puro-*HRAS*^{V12} vector and infected cell populations were selected in puromycin [9]. Constitutive knockdown of Beclin 1 was achieved in a glioblastoma cell line U251 using a shRNA construct as previously reported [10]. All cultures were maintained in a 37 °C incubator with 5% CO_2. RNAi-mediated inhibition of Beclin 1 gene expression was conducted by transfecting small interfering RNA (siRNA, 5′-GGUCUAAGACGUCCAACAA-3′, Invitrogen) with Oligofectamine (Invitrogen). Cells were analyzed 48 h later. A scrambled siRNA (5′-UUCUCCGAACGUGUCACGU-3′, Qiagen) was used as a negative control.

2.3. Immunoblot assay

Cells were washed in PBS and lysed in RIPA buffer. Forty micrograms of protein were separated by SDS–PAGE and transferred to PVDF membranes. The membranes were blotted with the indicated primary and secondary antibodies and developed with SuperSignal West Pico chemiluminescent substrate (Pierce). Images were obtained and analyzed using Kodak Image Station 4000 MM and the companion software (Carestream Health, Inc.).

2.4. Analysis of cell death

Cell viability was determined using propidium iodide (1 μg/mL) staining, or by crystal violet assay. The OD values were measured using a Tecan spectrometer at 540 nm. Apoptotic cells with condensed or fragmented nuclei were determined with Hoechst 33342 (5 μg/mL) staining. Analysis of the effector caspase activity was performed using 30 μg of proteins and 20 μM of fluorescent substrates (Ac-DEVD-AFC). The fluorescence signals were detected by a fluorometer (Tecan GENios) at 400/510 nm (excitation/emission), as described previously [11].

2.5. Statistical analysis

All experiments have been performed at least three times. Data shown are the mean ± SD from three experiments and were subjected to Student's *t*-test or one-way ANOVA followed by Holm–Sidak post hoc analysis (SigmaStat 3.5). A level of $p < 0.05$ was considered significant.

3. Results and discussion

In the classical autophagy pathway, the Atg5–Atg12–Atg16 complex is required to conjugate LC3I to phosphotidylethanolamine, forming LC3II, and thus is indispensible for autophagsome formation. Autophagy is hampered in Atg5-deficient cells. Loss of autophagy function in normal cells could subject them to oncogenesis [12,13]. It is not clear, however, the impact of this loss on the survival of the subsequently formed tumor cells.

To address this issue, we transformed wild-type and Atg5-deficient MEFs with oncogenic H-Ras. Growth of both cell lines in soft agar and colony formation indicated the establishment of anchorage-independent growth, a key index of oncogenic transformation (data not shown). Interestingly, Atg5-/- MEFs formed fewer colonies than the wild-type cells upon transformation, suggesting that autophagy would facilitate transformation as also noted in other studies [14–17]. We then treated these transformed cells with the proteasome inhibitor, bortezomib, for 16 h, and found that the Ras-transformed cells were more sensitive to proteasome inhibition than their non-transformed parental cells, with a higher caspase-3 activity and more cleaved caspase-3 (Fig. 1A and B). Cell death was also increased in the transformed cells as measured by both membrane permeability and nuclear morphology (Fig. 1C and D). Deletion of Atg5 led to the lack of bortezomib-stimulated conversion of LC3I to LC3II in Atg5-/- MEF (Fig. 1B), consistent with hampered autophagy. However, Ras activation in autophagy-deficient Atg5-/- MEF cells did not render the cells more susceptible to proteasome inhibitors than the wild type cells, as it would be expected based on previously established notion that autophagy would be required for an improved survival under proteasome inhibition [7]. In fact, both the levels of caspase activity and apoptosis were lower in transformed Atg5-/- cells than their wild-type counterparts following bortezomib treatment, as in the non-transformed condition (Fig. 1A, C). Overall, this set of experiments suggested that cells that were deficient in autophagy before being transformed were actually more resistant to proteasome inhibition, whereas cells that became autophagy-deficient after transformation would show increased death [7]. It is likely that cells can adapt to the permanent deficiency of autophagy so their survival under stress would not be affected by this mechanism even in the transformed status.

To further address the notion that short-term, but not long-term suppression of autophagy can make tumor cells more sensitive to proteasome inhibition, we investigated a different set of tumor cells. Beclin 1 (Atg6) interacts with the class III PI3 kinase to initiate autophagy. Knock-down of Beclin 1 can significantly reduce autophagy. In an autophagy-competent U251 glioblastoma cell line, use of a proteasome inhibitor, bortezomib, increased caspase-3 activity by two folds (Fig. 2A). Following an acute knock-down of Beclin 1 expression with a specific siRNA (Fig. 2C), an increased sensitivity to proteasome inhibition was observed, as manifested by a 5-fold increase of the caspase-3 activity (Fig. 2A). Cell death, as determined by positive PI staining, also revealed a higher sensitivity in cells with acute Beclin 1 knock-down (Fig. 2B). Hence acute Beclin 1 knock-down rendered the cell more sensitive to proteasome inhibitor-induced caspase activation and death. This finding was consistent with our previous work with other types of

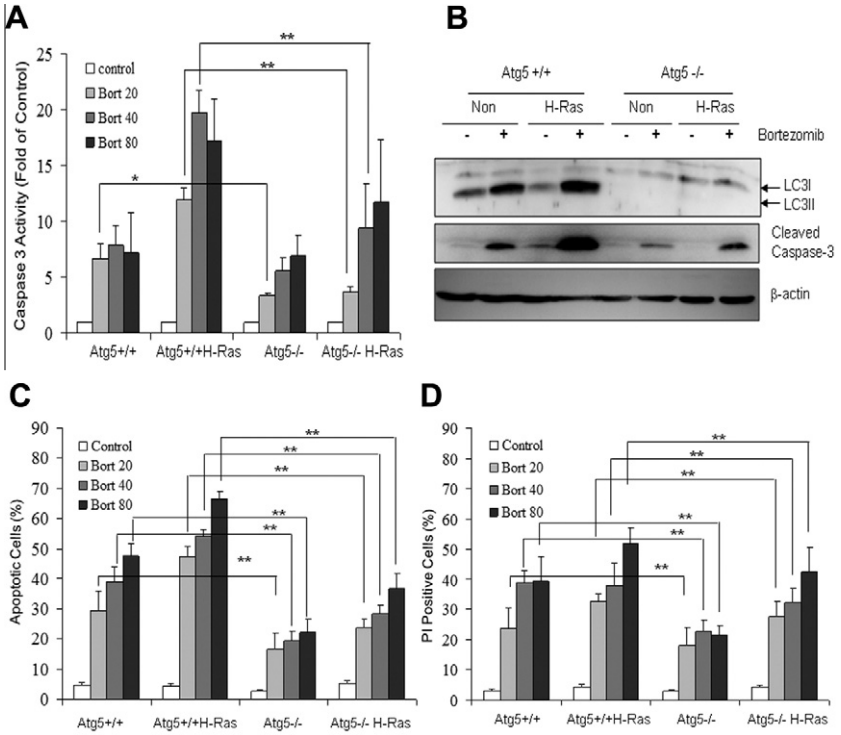

Fig. 1. Cells with constitutive autophagy deficiency did not become more sensitive to proteasome inhibitors when transformed. Wild-type and Atg5-/- MEFs were transformed with activated H-Ras and then treated with bortezomib (Bort) at different dose (nM) for 16 h. Caspase activation was determined by in vitro cleavage assay (A) or by western blotting (B), in which a cleaved caspase-3 fragment was detected. Cell death was measured by Hoechst 33342 staining for fragmented or condensed nuclei (C) and by PI staining (D); $^{*}P < 0.05$, $^{**}P < 0.01$.

cancer cells [7]. In a different set of experiments, U251 cells were transfected with a Beclin 1-specific shRNA construct that would allow constitutive expression of the inhibitory siRNA. Selection for cells expressing this shRNA construct allowed the establishment of an U251 cell line with consitutively low level of Beclin 1 [10] (Fig. 2C). Bortezomib treatment of this cell line did not reveal an increased cell death, as measured by caspase-3 activity (Fig. 2D) and cell viability (Fig. 2E). In these experiments, compared with wild-type cells, cells with acute Beclin 1 knock-down were more sensitive to proteasome inhibitor-induced apoptosis, but cells with constitutive knock-down were no more sensitive to the same treatment, but were more refractory. These results suggest that transformed cells with constitutively suppressed autophagy can adapt to this autophagy deficiency, and become less dependent on autophagy for survival. However, acute knock-down did not result in such an adaptation.

Autophagy defends cells against metabolic stress, which can be a feature of transformed cells. Autophagy and oncogenic transformation can have intricate relationships that affect each other at several levels. Expression of either H-Ras or K-Ras in immortalized baby mouse kidney (iBMK) cells increased the basal level of autophagy in nutrient-rich conditions, and enhanced the tumorigenecity of the transformed cells [15]. Loss of Beclin 1 and Atg5 rendered these cells more susceptible to ischemia and DNA damage [18]. In addition, Ras transformation can upregulate autophagy genes, which then affect cell proliferation at defined time points that would have either negative or positive impact [16,17,19,20]. Furthermore, adhesion-independent transformation in Ras-activated cancer cells could be driven by autophagy-facilitated glucose metabolism [14,16]: glucose uptake and lactate production was reduced in Atg5-/- MEF compared with wild-type MEF after transformation. Finally, in our early experiments with ovarian cells transformed with H-Ras and K-Ras [7] we found that they manifested an increased autophagy level, reflecting the dependency of these cells on this catabolic pathway, and that such an elevation of autophagy has made the cells more susceptible to apoptotic death when stressed with autophagy inhibition. We have also found that the transformation status of the cells determines their susceptibility to the combined inhibition of autophagy and proteasome. Therefore, the mutual dependence of oncogenic transformation and autophagy has become an attractive target for therapeutic considerations.

Considering their different roles in cellular metabolism, a combination of autophagy and proteasome inhibitors can be a more effective strategy to kill tumor cells than a single-drug regimen. However, this strategy would work only in tumor cells whose autophagy function remains intact and would also dependent on the transformation status. Notably, tumor cells with autophagy-deficiency prior to transformation had a different response to proteasome inhibitors compared to tumor cells with an acute suppression of their autophagy function. We showed that cells with an intrinsic suppression of autophagy (loss of Atg5) did not become more susceptible to proteasome-induced apoptotic death after transformation. Furthermore, susceptibility to proteasome inhibition was not increased in a glioblastoma cell line with long-term suppression of autophagy (constitutive knock-down of Beclin 1). The data suggest that although transformed cells with a deficiency in autophagy is less proliferative than autophagy-competent transformed cells, they can adapt to the autophagy-deficient conditions, and no longer depend on this pathway to mitigate the metabolic and other types of stress. Thus these cells could be more resistant to chemotherapy.

Taken together, autophagy seems to be more important for tumor cells to survive than for normal cells. Autophagy inhibition can thus be a therapeutic strategy for autophagy-intact tumors. However, resistance to autophagy suppression can occur in certain tumors, particularly in those tumors that arise because of the loss of autophagy capability at the very beginning. These tumor cells may

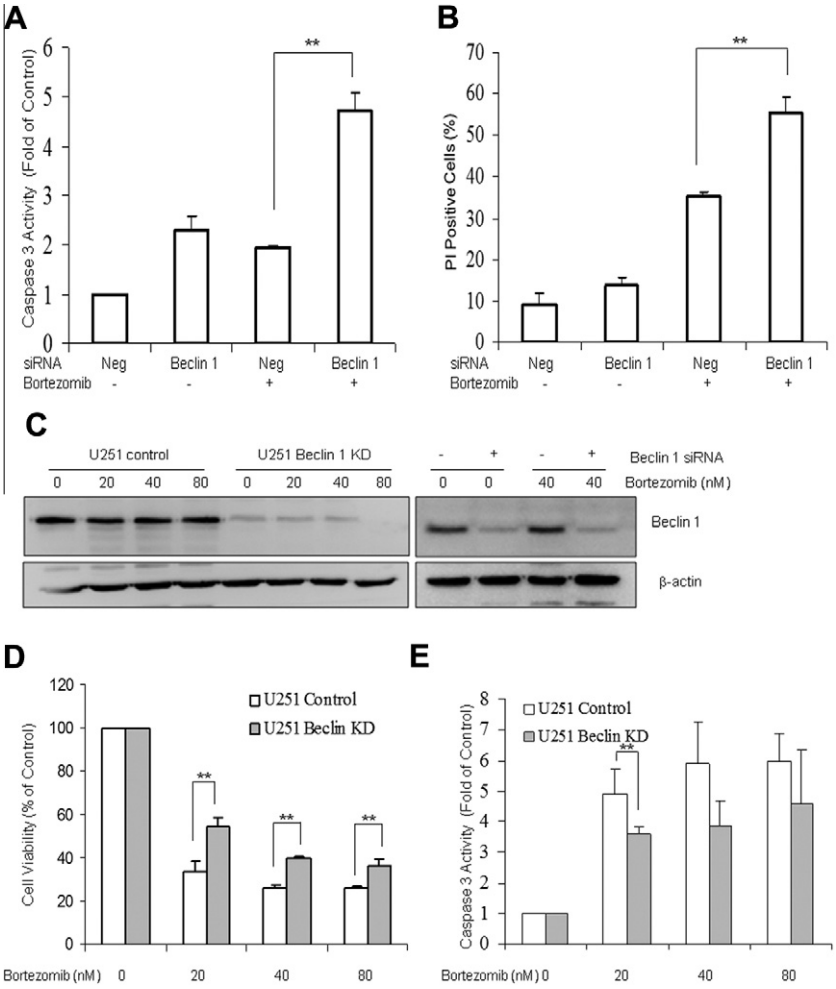

Fig. 2. Acute suppression of autophagy, but not constitutive autophagy deficiency, renders tumor cells more sensitive to proteasome inhibitors. Beclin 1 was acutely knocked down in the wild-type glioblastoma cell line (U251) by transfection of specific siRNA for 48 h (A and B). Cells were treated with bortezomib (40 nM) for another 24 h before caspase activation (A) and cell death (B) were analyzed. U251 cells with or without constitutive or acute knock-down of Beclin 1 (C and E) were treated with bortezomib at different doses (nM) for 48 h before crystal violet assay was carried out to examine cell viability (D), and for 24 h before the caspase-3 activity was determined (E); $^{**}P < 0.001$.

adopt alternative metabolic pathways, rendering them independent from autophagy-mediated protection. Suppression of autophagy would not be useful to inhibit these tumors. Thus it would be important to determine the status of autophagy of cancer cells before the application of a combinational strategy involving autophagy inhibition. It would also be important to investigate if a different metabolic target other than the autophagy machinery could be attacked in these autophagy-deficient cancer cells.

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