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Targeted Activation of Innate Immunity for Therapeutic Induction of Autophagy and Apoptosis in Melanoma Cells

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SUMMARY

Inappropriate drug delivery, secondary toxicities, and persistent chemo- and immunoresistance have traditionally compromised treatment response in melanoma. Using cellular systems and genetically engineered mouse models, we show that melanoma cells retain an innate ability to recognize cytosolic double-stranded RNA (dsRNA) and mount persistent stress response programs able to block tumor growth, even in highly immunosuppressed backgrounds. The dsRNA mimic polyinosine-polycytidylic acid, coadministered with polyethyleneimine as carrier, was identified as an unanticipated inducer of autophagy downstream of an exacerbated endosomal maturation program. A concurrent activity of the dsRNA helicase MDA-5 driving the proapoptotic protein NOXA resulted in an efficient autodigestion of melanoma cells. These results reveal tractable links for therapeutic intervention among dsRNA helicases, endo/lysosomes, and apoptotic factors.

INTRODUCTION

Melanoma remains a prototype of solid cancers with increasing incidence and extremely poor prognosis at advanced stages (Jemal et al., 2008). Considerable effort has been devoted to the identification of molecular determinants underlying melanoma chemo- and immunoresistance (Chin et al., 2006; Gray-Schopfer et al., 2007). Still, the average survival of patients with inoperable disseminated metastases is less than 10 months (Tawbi and Kirkwood, 2007). High throughput histogenetic and functional studies have revealed complex mechanisms associated with treatment failure (Fecher et al., 2008). These range from increased expression of drug pumps and detoxification enzymes to a pleiotropic potentiation of key survival pathways (Fecher et al., 2008; Gray-Schopfer et al., 2007; Soengas and Lowe, 2003). In addition, apoptotic programs involving the mitochondria, the endoplasmic reticulum, or death receptors are invariably ineffective in vivo (Hersey and Zhang, 2008; Soengas and Lowe, 2003). Consequently, current anticancer drugs either do not reach their target(s) in a productive manner or have to be

SIGNIFICANCE

Melanoma cells accumulate multiple genetic and epigenetic alterations. Still, they still remain highly sensitive to dsRNA mimics, shown here for the synthetic molecule pIC. However, the delivery vehicle is critical. PEI, a polycation that favors endosomal uptake and cytosolic release, was able to shift the mode of action of pIC from a transient innocuous transcriptional program to persistent cycles of fusion events involving a sequential recruitment of Rab7 (a small GTPase), LC3 (autophagosome marker), and lysosomes. A convergent mechanism of cellular stress was found driven by MDA-5 and involving an efficient NOXA-dependent caspase activation. Selective antitumor activity of $[pIC]^{PEI}$ in vivo further supports cytosolic dsRNA sensors as viable targets for drug development in melanoma.

administered at dosing schedules that result in unbearable toxicities to normal cellular compartments (Tawbi and Kirkwood, 2007). Similarly, melanomas have an inherent ability to bypass or overcome antitumoral activities of immunomodulators (Ilkovitch and Lopez, 2008; Tormo et al., 2006; Verma et al., 2008).

Autophagy, and in particular macroautophagy, which involves the sequestration of bulk cytosolic components in autophagosomes for subsequent lysosomal degradation (Xie and Klionsky, 2007), is an understudied process in melanoma. The clinical relevance of macroautophagy (herein referred to as autophagy for simplicity) stems from its potential to protect cells against a variety of intracellular and extracellular stress signals and favor tumor development (Mathew et al., 2009; Mizushima et al., 2008). Paradoxically, autophagy has also been associated with cell death (Kroemer et al., 2009). Thus, excessive or persistent autophagy can promote cell killing by depletion of key organelles (e.g., endoplasmic reticulum or mitochondria), rewiring of survival signals, deregulation of lysosomal enzymes, and/or activation of caspase-dependent apoptotic programs (Eisenberg-Lerner et al., 2009; Hoyer-Hansen and Jaattela, 2008; Maiuri et al., 2007; Xie and Klionsky, 2007). Given these pro- and anti-apoptotic roles of autophagy, it is unclear whether this program could be a viable target for drug development (Kroemer and Levine, 2008; Rubinsztein et al., 2007; Scarlatti et al., 2009).

Autophagy genes can also have pleiotropic roles in the immune system (Virgin and Levine, 2009). Thus, autophagy can modulate antigen presentation, inhibit or potentiate interferon responses, and display critical functions in the clearance of intracellular viral and bacterial pathogens (Levine and Deretic, 2007; Sanjuan and Green, 2008). Typically, these responses are engaged to protect infected cells or the host (Virgin and Levine, 2009). The precise mechanisms underlying this immune autophagy are not well defined. Membrane-bound pattern recognition receptors of the Toll-like receptor (TLR) family (particularly TLR-3, TLR-4, and TLR-7) can favor pathogen sequestration in autophagosomes (Delgado and Deretic, 2009; Levine and Deretic, 2007). These TLRs have a restricted expression pattern, being enriched in cells of the immune system, such as macrophages and dendritic cells (Paulos et al., 2007; Wenzel et al., 2008). Whether melanoma cells have other sensors of viral pathogens that can be engaged to induce autophagy and cell death is unknown.

Here, we have assessed the interplay between autophagy and apoptosis in the context of tumor cell-selective elimination of melanoma cells.

RESULTS

Identification of Autophagosome Inducers in Melanoma Cells

Melanoma cells stably expressing the autophagosome marker LC3 fused with GFP (Klionsky et al., 2008) were used to screen for autophagy inducers among commercially available chemotherapeutic drugs and immunomodulators. To improve intracellular delivery, cationic carriers, e.g., polyethyleneimine (PEI), were added to DNA- or RNA-based agents (Bieber et al., 2002). The initial screen was performed with the SK-Mel-103 cell line. Subsequent validation studies were performed using a panel of nine human metastatic melanoma cell lines of diverse genetic background (see Table S1 available online), as well as the well-known B16 mouse melanoma cells. Primary skin melanocytes, keratinocytes, and fibroblasts were included as controls for normal cells. We also tested drug response in mouse embryonic fibroblasts (MEFs) expressing or deficient for the autophagy factor Atg5 to provide a genetically controlled model to assess classical autophagy programs (Salazar et al., 2009).

Multiple drugs were found to promote focal GFP-LC3 fluorescence emission without significantly affecting cell viability (A.C. and M.S.S., unpublished data). Among prodeath agents, the classical double-stranded RNA (dsRNA) mimic polyinosine-polycytidylic acid (pIC; Wenzel et al., 2008) complexed with PEI ($[pIC]^{PEI}$) induced a potent accumulation of GFP-LC3 foci (Figure 1A). About 50% of cells treated with low doses (0.5–1 μg/ml) of $[pIC]^{PEI}$ showed GFP-LC3 staining within 2–4 hr. These results were intriguing as pIC had been linked to autophagy in immune cells (Delgado et al., 2008), but not in the context of tumor cell death. Therefore, we focused on the identification of the cellular machinery that sensed and executed the response of melanoma cells to $[pIC]^{PEI}$.

Consistent with autophagy, $[pIC]^{PEI}$ induced electrophoretic mobility changes of the endogenous LC3, which are characteristic of lipidation of this protein during autophagy (Figure 1B). $[pIC]^{PEI}$ also lead to LC3 foci formation in oncogenically transformed MEFs, an activity that required Atg5 (Figure 1C, upper panels). In fact, inhibition of LC3 redistribution in $Atg5^{-/-}$ cells treated with $[pIC]^{PEI}$ was as prominent as with rapamycin (Figure 1C), a classical autophagy inducer (Noda and Ohsumi, 1998). However, rapamycin and $[pIC]^{PEI}$ were not equivalent. LC3 foci were transient in melanoma treated with rapamycin, but sustained with $[pIC]^{PEI}$ (Figure 1D). In addition, rapamycin, but not $[pIC]^{PEI}$, inhibited the mTOR pathway as visualized by monitoring the expression of the phosphorylated S6 kinase (Figure 1E).

Electron microscopy provided independent evidence of autophagosome/autolysosome formation driven by $[pIC]^{PEI}$. Thus, large mutivesicular structures (>500 nm diameter; Figure $1F_d$) were found following uptake of $[pIC]^{PEI}$ nanoparticles in melanoma cells (Figure $1F_b$). This uptake was probably facilitated by the known activity of PEI to coat genetic material and favor endocytosis (Boussif et al., 1995; Kopatz et al., 2004). Importantly, neither naked pIC nor PEI alone was able to induce autophagosome formation (see Figure S1 for representative examples). Together, these results suggest an mTOR-independent autophagy driven by pIC that requires an appropriate cellular delivery method.

Selective Melanoma Cell Death after $[pIC]^{PEI}$ -Driven Autophagy

Notably, in all melanoma cell lines tested in this study, the early activation of autophagy by $[pIC]^{PEI}$ was invariably followed by cell death (Figure 1G and Table S1). In contrast, melanocytes retained their viability and did not display markers of autophagy (Figures S2A–S2C). Interestingly, although complexed efficiently with PEI, dsDNA, the immunogenic variant ds-BDNA (Ishii et al., 2006), or other dsRNA molecules such as pA:U had no obvious impact on melanoma physiology (Figure S2D and data not shown). These results are consistent with the known superior immunomodulatory efficacy of pIC over other dsRNA mimics (Alexopoulou et al., 2001).