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Article Addendum

Autophagy-driven cell fate decision maker

Activated microglia induce specific death of glioma cells by a blockade of basal autophagic flux and secondary apoptosis/necrosis

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Programmed cell death is classified into apoptosis and autophagic cell death. The extensive crosstalk that occurs between these two types of death often prevents a clear identification of the leading death mechanism in a given experimental system. An accurate assessment of the type of death at work is of crucial relevance for the design of efficient cancer therapies aiming at eliminating tumor cells. Indeed, accumulating evidence indicates that resistance of tumor cells to apoptosis can be overcome by induction of autophagy. The latter would thus seem to represent an ideal strategy for eliminating certain tumor cells, except for the fact that autophagy induction may also contribute to cell survival.

It therefore is of paramount importance to clarify the mechanistic links between autophagy and apoptosis as well as the nature of autophagy-dependent cell death. We recently reported that glioma cells resistant to death ligands were killed by the supernatant of activated microglia. What at first glance seemed to be apoptosis turned out to be autophagy-dependent cell death resulting from a blockade in the autophagic flux. This blockade most likely occurs at the level of lysosome recycling. We hypothesize that this autophagy-dependent process leads to either apoptosis or necrosis depending on the extent of lysosomal permeabilization and on the relative contribution of other cellular compartments. Autophagy therefore appears in our model as a cell-fate decision maker, not as a cell death execution pathway.

Macroautophagy (referred to hereafter as autophagy) is a puzzling Janus that plays a determinant role in cell fate. Originally described for its contribution to cell survival, increasing evidence indicates that, beside apoptosis, it occupies a very important seat in the orchestra of programmed cell death mechanisms.[1-3] The fact that autophagy leads to these two opposite fates—life and death—raises questions about the nature of what we refer to as "autophagic cell death" and about the mechanisms that underlie it.[4-8] Is death caused by an exacerbation of autophagy leading to cell self-digestion? Is it caused by a deficiency in autophagy leading to accumulation of aged organelles or proteins? If cell survival-supporting functions can be turned into cell death-inducing functions, how are these transitions regulated, and what are the molecular switches? The answer to these questions is of utmost importance not only for our understanding of this biological phenomenon but also because of its high relevance for cancer therapy. "Autophagic cell death" indeed has been reported as an alternative and efficient death pathway in apoptosis-resistant tumor cells and hence represents a promising target for new therapies.[9-13] Elucidation of the mechanisms that underlie "autophagic cell death" is however complicated by the extensive crosstalk between apoptosis and autophagy which raises the question of the molecular links between these two types of cell death.[14-16] At the experimental level, it is therefore essential to achieve a correct identification of apoptosis and autophagy in a given experimental setup in order to properly and timely assess their specific involvement in the death process. In that regard, special caution must be paid to the experimental techniques employed to assess cell death. Too often only a few methods will be used, resulting in an improper characterization of cell death and hence in possible erroneous interpretations of the data.

We recently reported the characterization of the antitumor cytotoxicity exerted by the supernatant of activated microglia, the brain macrophages, towards glioma cells that were resistant to various death inducers.[17] The first set of methods we employed (video-microscopy, annexin V labelling, determination of DNA loss by the Nicoletti method) provided data that were strongly suggestive of apoptotic cell death. If we had stopped our investigation at that point, we would have come to a fully wrong conclusion. Indeed, whereas the use of the pan-caspase inhibitor z-VAD-fmk prevented DNA loss, it was inefficient in rescuing cells from death. This suggests that apoptosis was not the dominant pathway that decided the final cell fate. Caspases indeed were not crucial for death induction and played a secondary role in final degradation processes that led to DNA fragmentation. In that regard, we would like to stress the fact that DNA loss should be assessed simultaneously with membrane permeability in order to avoid wrong interpretations of the type of cell death that dominates in a specific experimental setup.

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We next performed a series of experiments in order to assess a possible involvement of autophagy in this cell death process. Electron microscopy, analysis of the content of dying cells in acidic (acridine orange labelled) vesicles, and the use of 3-methyladenine (3-MA), an inhibitor of autophagosome formation, provided the first evidence for autophagy ongoing in the dying cells. Moreover, cell death was strongly inhibited by 3-MA, speaking in favor of autophagic death or, to be more cautious at that step of our investigations, of an autophagy-dependent death. A further careful examination of autophagic flux, that truly reflects autophagic activity,[3,18] indicated that the microglia supernatant induced a blockade of the autophagic flux that was constitutively present in glioma cells. The actual role of this blockade in death induction by microglia—its nature and its regulation—are currently under investigation.

The model shown in Figure 1A summarizes our observations and current hypotheses. We observed and characterized a cell death mechanism which depends on an active autophagic flux. This cell death process involves the accumulation of acidic vesicles, suggesting a functional fusion of autophagosomes with lysosomes. We therefore hypothesize that blockade of the autophagic flux occurs at a later step, most probably lysosomal fission. In the context of active basal autophagosome formation, a blockade in lysosomal fission would lead over time to a massive accumulation of hybrid autolysosome organelles and to a deficient lysosomal degradation pathway.[19-22] Such huge vacuoles were often observed in glioma cells dying upon treatment with the microglia supernatant (Fig. 1B). An unstable, growing (auto-) lysosome represents a great danger to the cell since it could lead to permeabilization and to the release of the lysosomal content into the cytosol.[23-25] Among the lysosomal proteases, cathepsins B and D have been described for their involvement in cell death mechanisms.[26,27] With regard to our experimental system, they represent possible candidates linking an autophagy-dependent cell death with secondary caspase activation. The extent of lysosomal permeabilization has been reported to correlate with the decision of whether the cell dies through apoptosis or necrosis. Low lysosomal permeabilization could lead to a specific externalization of cathepsins and activation of mitochondrial proapoptotic mechanisms. A higher lysosomal rupture could lead to an extensive release of general lysosomal enzymes and the induction of necrotic cell death.[19,25] We hypothesize that in our experimental system, autophagy drives the cells into a lysosome-mediated death process; this cell death ends as apoptosis or necrosis, depending on the extent of lysosomal permeabilization and/or on the cellular context in terms of relative contribution of different organelles, such as mitochondria.[22] One implication of this hypothesis would be that a given tumor cell (line/population) may have a "preferred way to die" that is dictated by its cellular context and not (or not only) by the death stimuli. In that respect, it is interesting to note that the two mouse glioma cell lines used in our experimental system responded to an increase in their content of acidic vesicles not only to the supernatant of activated microglia but also to various chemotoxic drugs (etoposide, staurosporine, cisplatin; data not shown), suggesting that an autophagy-dependent death was their "preferred way of dying."

A final note should be added regarding the molecular basis of this blockade of autophagic flux. The study of (auto)phagosome-lysosome fusion has received much attention and its molecular basis is well characterized. However, the still neglected step of lysosomal fission or recycling[28,29] should receive as much attention as the fusion step. Indeed autophagy is a dynamic flux and as such it

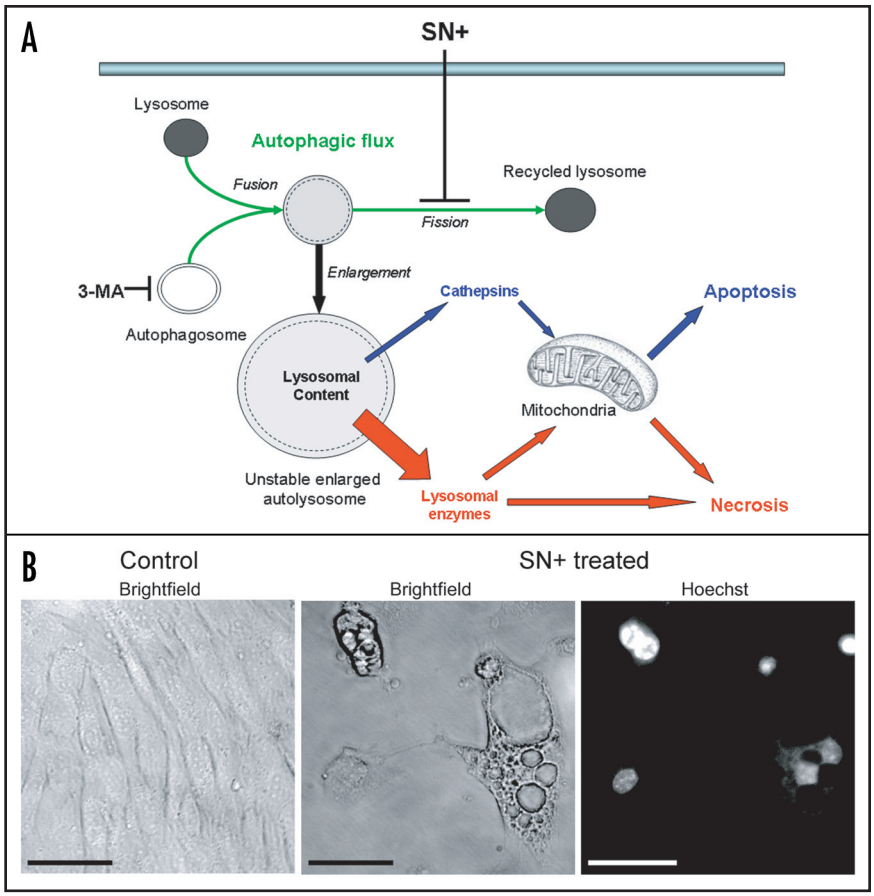

Figure 1. (A) Hypothetical model of autophagy-driven cell death decision maker. A basal autophagic flux leads to the formation of autophagosomes, a first step that can be prevented by the use of 3-methyladenine (3-MA), making the whole process autophagy-dependent. Mature autophagosomes fuse with lysosomes to form hybrid autolysosome organelles where degradation takes place. After complete processing, the autolysosome undergoes fission in order to recycle the lysosomal compartment. If this fission process is interrupted, i.e., by the effect of factors present in the supernatant of (LPS and IFNγ) stimulated microglia (SN+) and further autophagosomes continue to fuse, the autolysosome grows until reaching an unstable size that could lead to permeabilization and release of lysosomal factors. A low, selective release of cathepsins may trigger mitochondrial mechanisms that will lead the process towards an apoptotic cell death. A higher lysosomal permeabilization would allow general lysosomal enzyme externalization, leading to necrotic cell death. Although cell death is autophagy-dependent, the final type of cell death will be dictated by the extent of lysosomal permeabilization and the relative contributions of the lysosomal and mitochondrial compartments. (B) Brightfield microphotographs of MT539MG glioma cells incubated for 2 days with either medium (control, left) or SN+ (middle and right). The middle panel shows a cell containing huge cytoplasmic vesicles and several dead cells; the right panel indicates the localization of Hoechst-labelled nuclei in these treated cells. Bar: 50 μm.

requires a recycling of the lysosomal compartment in order to ensure cellular homeostasis. The understanding of the molecular basis of this recycling process may have profound implications for the field of autophagy and autophagy-dependent cell death. This recycling process moreover represents a target of interest for cancer therapy, especially in the case of cancer types that take advantage of an increased basal autophagic flux for their growth and survival.

In conclusion we have shown that in our experimental system, autophagy acted as a decision maker of cell death rather than as an executor of cell death. Further investigations shall demonstrate whether this is indeed the main role of autophagy in cell death.

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