Metformin inhibits melanoma development through autophagy and apoptosis mechanisms

Metformin is the most widely used antidiabetic drug because of its proven efficacy and limited secondary effects. Interestingly, recent studies have reported that metformin can block the growth of different tumor types. Here, we show that metformin exerts antiproliferative effects on melanoma cells, whereas normal human melanocytes are resistant to these metformin-induced effects. To better understand the basis of this antiproliferative effect of metformin in melanoma, we characterized the sequence of events underlying metformin action. We showed that 24 h metformin treatment induced a cell cycle arrest in G0/G1 phases, while after 72 h, melanoma cells underwent autophagy as demonstrated by electron microscopy, immunochemistry, and by quantification of the autolysosome-associated LC3 and Beclin1 proteins. In addition, 96 h post metformin treatment we observed robust apoptosis of melanoma cells. Interestingly, inhibition of autophagy by knocking down LC3 or ATG5 decreased the extent of apoptosis, and suppressed the antiproliferative effect of metformin on melanoma cells, suggesting that apoptosis is a consequence of autophagy. The relevance of these observations were confirmed in vivo, as we showed that metformin treatment impaired the melanoma tumor growth in mice, and induced autophagy and apoptosis markers. Taken together, our data suggest that metformin has an important impact on melanoma growth, and may therefore be beneficial in patients with melanoma.

Subject Category: Cancer

Cutaneous melanoma deriving from the transformation of pigment-producing melanocytes is one of the most lethal cancers among young adults. Its incidence has increased at a dramatic rate during the last decades. Melanoma progression is characterized by an initial radial growth phase, encompassing in situ and minimally invasive tumors. This phase is followed by the development of vertical growth phase, which has been postulated to be the first point at which the tumor gains metastatic ability. Indeed, melanoma has a high capability of invasion and rapid metastasis to other organs. The prognosis of metastatic melanoma is extremely pejorative, as the various protocols of chemotherapy or immunotherapy have not shown, for the moment, real survival benefit. In addition to active prevention and early detection of melanomas, it appears necessary to develop new approaches enabling the discovery of new molecular target candidates for specific biotherapy treatment of this disease.

To this purpose, we have been interested in studying the effect of the oral antidiabetic drug, metformin, on melanoma. Metformin belongs to the family of biguanide and is the most widely used antidiabetic drug in the world. This effect of metformin on glucose homeostasis has been explained through reduced hepatic gluconeogenesis and increased glucose uptake in skeletal muscles. This drug has the major clinical advantage of not inducing hypoglycemia and being tolerated very well. It is associated with only very low incidence of lactic acidosis (1/30 000) predominantly in patients with altered kidney or liver functions. The mechanism through which metformin reduces hepatic glucose production requires LKB1, which controls the AMPK (AMP-activated protein kinase)/mTOR (mammalian target of rapamycin) pathway and neoglucogenic genes.

Metformin action on the AMPK/mTOR pathway leads to reduced protein synthesis and cell proliferation. These observations have given the impetus to numerous studies on the role of metformin in the regulation of tumor cell proliferation and apoptosis. Encouraging results emerged from these studies indicating that metformin can potentially be used as an efficient anticancer drug in various neoplasms such as prostate, breast, lung and pancreas cancers. These results were confirmed by retrospective epidemiological studies that reported a decrease in cancer risk in diabetic patients treated with metformin. Importantly, a recent work of Nakajima lab demonstrates that metformin diminishes the formation of rectal aberrant crypt foci, a marker of colorectal cancer, in non-diabetic patients. Despite

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Abbreviations: AMPK, AMP-activated protein kinase; PBS, phosphate-buffered saline; DMEM, Dulbecco’s Modified Eagle’s Medium; FCS, fetal calf serum; PARP, poly(ADP-ribose) polymerase; pRB, retinoblastoma protein

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compelling evidence of a role of metformin as an anticancer drug, its mode of action in cancer remains unelucidated. In a few studies, metformin induces apoptosis in cancer, and in one study performed on colon cancer, metformin triggers autophagy. Undoubtedly, in cancer there are multiple functional relationship reported between the apoptosis and autophagy, and these processes separately or/and jointly seal the fate of the cell. Thus, apoptosis or/and autophagy are interesting mechanisms to induce cancer cell death.

In this work, we wished to study the effect of metformin on melanoma cell viability, and to further investigate the molecular mechanisms by which metformin exerts its action on melanoma cells.

Results

Metformin exerts a deleterious effect on melanoma cell viability. Metformin has been shown to affect the proliferation of several human cancer cell lines derived from the prostate, colon, gliomas and breast, in the 1–10 mM range. To investigate the effect of metformin on melanoma cells, we treated different human melanoma cell lines (A375, WM9, SKMel28 and G361) with metformin, and monitored cell number. As shown in Figure 1a, 72 h exposure of cells to various concentrations of metformin led to dose-dependent decrease in cell number. In contrast, human melanocytes were resistant to metformin treatment. In addition, metformin induced dose-dependent decrease in cell number of two melanoma cell samples freshly isolated from patient tumors (Figure 1b). Time-course experiments showed a rapid effect of 10 mM metformin, with an almost immediate and important decrease of cell number in comparison to phosphate-buffered saline (PBS) control (Figure 1c).

Metformin induces autophagy. To elucidate whether AMPK activation is modulated in response to metformin, we studied the phosphorylation at Thr-172 of AMPK-α (AMPK T172) as a read-out of its activation state. Figure 2a shows that metformin induced a dose-dependent increase of AMPK T172 phosphorylation in A375 melanoma cell lines. We next examined the effect of metformin on the activity of mTOR, a downstream effector of AMPK. We observed a net decrease in the phosphorylation status of mTOR and S6 Ribosomal protein, suggesting a blockade of the mTOR pathway. As the mTOR pathway is a central regulator of multiple cellular responses to nutrient and growth factor signaling, including autophagy, we analyzed whether metformin might induce characteristic hallmarks of autophagy. During autophagy, LC3-I is converted to LC3-II through the ATG4-dependent insertion of a phosphoethanolamine moiety and recruited into the membrane of the forming phagophore, a double membrane required for the recycling of protein aggregates and organelles. Increased LC3 expression was detected for two doses of metformin. At the same concentrations, metformin also enhanced the expression level of Beclin 1, a protein required for the initiation of the formation of the autophagosome in autophagy. These data collectively strongly argue the induction of macroautophagy by metformin. Accordingly, electron microscopy images of A375 cells treated for 72 h with metformin showed typical images of autophagy including accumulation of numerous vesicles with distinct double membrane (Figure 2b). In addition, early autophagosomes sequestering cytosol, mitochondrion, or endoplasmic reticulum membranes were observed. By contrast, control melanoma cells (PBS) showed no autophagic vacuoles. Similar results were obtained with SKMel28 melanoma cells (Supplementary Figure S2).

In addition, following transfection of melanoma cells with RFP-LC3 construct, the formation of RFP puncta in cells treated with metformin was observed, whereas the untreated cells exhibited a diffuse distribution of red fluorescence in the cytoplasm and nucleus (Figure 2c).

The observation that metformin affected the lysosomal compartment was further substantiated by the increase of cathepsin B activity mediated by metformin at 72 h in A375 and SKMel28 cells (Figure 2d). Importantly, increased cathepsin B activity has been associated with the late steps of autophagy.

Metformin induces cell death. To determine whether autophagy mediated by metformin affects cell viability, we analyzed the percentage of cell death, by staining with propidium iodide (PI) in response to various metformin concentrations in A375 melanoma cells. Metformin induced a dose-dependent increase of cell death (Figure 3a). At a 10 mM metformin concentration, ~40% of the cells were dead. This result was comparable to that obtained with the proapoptotic effector, staurosporine.

Previous studies have demonstrated that coregulation of apoptosis and autophagy can participate in mammalian cell death. To analyze the relationships between apoptosis and autophagy in melanoma cell death, we treated A375 melanoma cells for different times with 5 and 10 mM metformin. To monitor apoptosis, we evaluated both the cleavage of DNA repair enzyme poly(ADP-ribose) polymerase (PARP; Figure 3b) and the activation of caspase 3 (Figure 3c). In parallel, we determined autophagy induction by measuring the expression of LC3-b, Beclin 1 and ATG5. As shown in Figure 3b, PARP cleavage was significantly increased after 72 h of treatment with 5 or 10 mM metformin. At the same time, a clear activation of caspase 3 was detected (Figure 3c). In the same period of time, we also detected an increased expression of three proteins involved in autophagy process such as LC3, Beclin1 and ATG5. The apoptosis inductor staurosporine was found to induce apoptosis but not autophagy. These results were confirmed by quantification of western blots using a Fuji Film, Multi Gauge Ver 3.0 software (Fuji Film, Tokyo, Japan) (Figure 3b, lower part). Taken together, these results indicate that metformin induces a concomitant induction of autophagy and apoptosis processes in melanoma cells, both of which are involved in cell death. To identify the event sequence of autophagy and apoptosis, we have performed another kinetics of 6 h intervals. Supplementary Figure S3 clearly shows that the increase of expression and the conversion of LC3 precede the cleavage of PARP, suggesting that the autophagy is set up before apoptosis.

In an attempt to identify the contribution of autophagy and apoptosis or a switch between the two processes to induce
cell death, we inhibited autophagy and apoptosis using LC3 or ATG5 small interfering RNA (siRNA) and pan caspase inhibitor Q-VD-OPH or caspase 3 siRNA, respectively. LC3 siRNA, which drastically reduced the expression of the protein (Figure 4a), prevented PARP cleavage (Figure 4a) and cell death induced by metformin (Figure 4b). The apoptosis inhibitor, Q-VD-OPH was capable of inhibiting PARP cleavage and cell death induced by metformin. In contrast,
Figure 2  Metformin triggers autophagy in melanoma cells. (a) A375 melanoma cells were treated with indicated concentrations of metformin for 72 h. Cell lysates were separated by SDS-PAGE and analysed by western blot using the indicated antibody. HSP60 and actin were used as loading control. One representative experiment of three is shown. (b) Electron microscopy images presenting ultrastructure in representative control (PBS) and 10 mM metformin-treated melanoma cells for 72 h. Arrowheads, autophagosomes. Nu, nucleus; M, mitochondria. (c) Representative confocal microscopy images of RFP-LC3 staining in melanoma cells expressing RFP-LC3 and stimulated with or without metformin. In parallel (right panel), number of cells with RFP-LC3 dots were scored on 50 transfected cells. (d) Melanoma A375 and SKMel28 cells were incubated without or with metformin for 72 h, and cathepsin B activity was evaluated in the presence or absence of CA-074Me. Results are expressed as arbitrary units (A.U.) per mg of proteins. Data are mean ± S.D. of representative experiment performed in triplicate. Significantly different from the corresponding control ***P < 0.001
Q-VD-OPH failed to impair LC3-II expression mediated by metformin. As expected, combination of both LC3 siRNA and Q-VD-OPH abolished autophagy, apoptosis and cell death triggered by metformin. Similar results were found with ATG5 and caspase 3 siRNA (Supplementary Figure S4).

The anti-melanoma effect of metformin is partially mediated by AMPK. The anticancer effects of metformin have been essentially attributed to its ability to activate the AMPK pathway. To evaluate the function of AMPK in the effects of metformin, we knockdown both α1 and α2 catalytic subunits of AMPK with previously validated siRNAs. As shown in Figures 5a and b, inhibition of α1 and α2 AMPK subunits partially prevented cell death, and PARP cleavage induced by 5 and 10 mM metformin. Further, AMPK siRNA inhibited partially LC3 cleavage at 5 mM metformin but not at 10 mM metformin. These observations suggest that metformin mediates its anti-melanoma effects via both AMPK-dependent and -independent pathways.

Metformin inhibits melanoma tumor development in mice. Finally, to assess a potential antineoplastic effect of metformin in vivo, A375 melanoma cells (2.5 × 10⁶) were injected subcutaneously in 6-week-old female athymic nude mice and treated 5 days later by injection of vehicle or metformin (2 mg/mouse/day) over a period of 3 weeks. Untreated control mice rapidly developed visible tumors, and dramatic tumor growth was observed throughout the course of the study (Figure 6a). In contrast, treatment of mice with metformin markedly attenuated the ability of cells to develop
metformin has anti-melanoma activity in vivo. Indeed, three of seven metformin-treated mice had no measurable tumors. Moreover, tumors derived from metformin-treated mice were consistently and significantly smaller than tumors from untreated control mice (Figure 6b). These data clearly demonstrate that metformin has anti-melanoma activity in vivo.

To confirm the molecular mechanisms involved in the antineoplastic effects of metformin in vivo, LC3 expression was studied by immunofluorescent staining on tumor sections from mice treated with vehicle or metformin (Figure 6c, upper part). Sections of tumors from mice treated with metformin show a dramatic increase in LC3 staining compared with sections of tumors from control mice injected with vehicle. In addition, LC3 puncta, reminiscent of autophagosome formation in tumors of mice treated with metformin, were visualized.

To determine the level of apoptosis in the tumors of mice treated with metformin, we performed a TUNEL assay on section of different tumors. We observed an increase in TUNEL-positive cells (green) in tumors sections from mice treated with metformin compared with tumor sections from mice treated with PBS.

In addition, to confirm these results, we made western blot from tumors of mice treated or not with metformin (Figure 6c, lower part). As expected, we showed an increase in expression and lipid conjugation of LC3b and cleavage of PARP in tumors of mice treated with metformin in comparison with control mice treated with PBS, suggesting that autophagy and apoptosis are also occurring in vivo.

Thus, the reduction of tumor volume and weight observed in metformin-treated mice seems to be, at least in part, related to the induction of autophagy and apoptosis.

Finally, we performed exactly the same experiment using a syngeneic tumor model in which B16 melanoma cells were injected in C57/Bl6 mice (Figure 6d). After 20 days of metformin treatment (2 mg/day/mouse), we observed a significant decrease in volume and weight of tumors for mice treated with drug compared with control mice, thereby confirming the antineoplastic effect of metformin in mice.

#### Discussion

The discovery of new therapeutic compounds is a very important challenge to treat advanced melanomas that are resistant to existing therapies. To this purpose, using in vitro and in vivo approaches, we demonstrate the potent anti-melanoma activity of the antidiabetic metformin. Interestingly, contrary to normal human melanocytes, drastic inhibition of cell viability mediated by metformin is observed in four different melanoma cell lines independent of the mutational status or melanoma development stage, and in two primary melanoma cell cultures freshly isolated from patients. These findings are consistent with results of Woodard et al., demonstrating AMPK activators AICAR and metformin reduced the proliferation of SKMel2 and SKMel28 melanoma cells.

Metformin action is mainly mediated by AMPK activation; however, several recent reports indicate that some effects of metformin in cancer cell lines could be mediated by an AMPK-independent pathway. In our model, AMPK silencing by siRNA (Figure 5) or inhibition of AMPK activation by pharmacological inhibitor (data not shown) inhibits only partially the effect of metformin on melanoma cell death, suggesting that the effects of this drug are mediated, at least in part, through an AMPK-independent mechanism. In addition, AMPK knockdown protects partially the cleavage of PARP but fails to block the induction of LC3-b at 10 mM metformin. This result indicates that AMPK-dependent component of anti-melanoma action of metformin could be autophagy-independent. However, we cannot exclude that the low residual AMPK expression is sufficient to mediate metformin effect on cell death. Noteworthy, treatment of normal human melanocytes that express endogenous AMPK with metformin did not affect cell viability (Figure 1a), suggesting that metformin action is restrained to transformed cell lines and likely reflects a tumor-specific regulation.

The AMPK/mTOR pathway is under the control of LKB1, a serine-threonine kinase acting as a tumor suppressor. A recent report demonstrates that oncogenic V600E B-RAF, a common somatic mutation found in malignant melanoma (50–70%), negatively regulates LKB1 to promote melanoma cell proliferation. In our model, LKB1 seems not to be involved in metformin’s anti-melanoma effect as G361 cells, which do not express LKB1, are also sensitive to the drug (Figure 1a). Accordingly, overexpression of a dominant negative form of LKB1 in melanoma cell lines did not prevent metformin sensitivity (data not shown).

Another protein that can mediate metformin effect is the tumor suppressor p53. Indeed, p53 functions as a transcriptional regulator of genes involved in cell cycle arrest,
autophagy and apoptosis pathways.\textsuperscript{25} In addition, the AMPK-p53 pathway may represent a cell cycle checkpoint in response to energy limitation. AMPK has been shown to phosphorylate p53 on Ser-15 to induce G0/G1 phase arrest.\textsuperscript{26} In our melanoma cell lines, p53 is not phosphorylated on Ser-15 in response to metformin (data not shown). Further melanoma cells harboring a mutated \textit{TP53} gene (SKMel28 cells) exhibited the same sensitivity to metformin as other melanoma cell lines harboring wild-type p53 (Figure 1a), indicating that p53 is not involved in metformin effects in our cellular model.

Several recent reports have highlighted that metformin induces cell cycle arrest of cancer cell lines.\textsuperscript{7} We observed here that the proportion of cells in G0/G1 was significantly increased in melanoma cells treated for 24 h with metformin, while apoptotic cells with fragmented DNA (sub G1) could not be observed at 24 h (Supplementary Figures S1a and b). Cell cycle arrest in G0/G1 phase is associated at molecular level with a dramatic reduction of cyclin D1 expression, a marked increase in the expression of cyclin-dependent kinase inhibitor p21Cip1/Waf1, and hypophosphorylation of pRb (Supplementary Figure S1c).

However, the antiproliferative effect of metformin cannot be exclusively explained by cell cycle arrest as a strong increase in cell death was detected for longer time of drug treatment (>48 h; Figure 3a). Autophagy is a catabolic process for the degradation and recycling of macromolecules and organelles, which is activated during stress conditions.\textsuperscript{27,28} Autophagy is initiated by the formation of double-membraned vesicles called autophagosomes, which fuse with lysosomes to form autophagolysosomes in which lysosomal hydrolases digest the vesicle contents for recycling.\textsuperscript{29} Autophagy is considered as a survival mechanism induced in adverse conditions to maintain cell integrity, but paradoxically, it is also involved in a particular mode of death called autophagic cell death or type II cell death.\textsuperscript{30} In our study, several compelling evidences indicate that metformin induces autophagy in melanoma cells (Figure 2). This finding is particularly interesting as until now, only one study has reported that metformin is able to induce autophagy in colon cancer.\textsuperscript{14} In this model and conversely to the present study, p53 seems to be required for autophagy after treatment with metformin. In agreement with this model, we observed that blockage of autophagy by siRNAs directed against LC3 and ATG5 (Figure 4a and Supplementary Figure S4) or pharmacological inhibition of autophagy by 3-methyl adenine (data not shown) promotes melanoma cell survival, suggesting that metformin does induce autophagic cell death.

In addition, we observed that long-term treatment with metformin induces apoptosis as monitored by caspase 3
activation and PARP cleavage (Figures 3b and c). This result is consistent with the fact that metformin has been previously shown to promote apoptosis in several solid tumor cell lines.7

To determine whether autophagy and apoptosis are linked mechanisms, we performed several experiments following inhibition of apoptosis and/or autophagy. Inhibition of caspase activation by Q-VD-OPh or caspase 3 siRNA did not dampen LC3-II accumulation nor the melanoma cell death in response to metformin (Figure 4 and Supplementary Figure S4), suggesting that metformin-mediated apoptosis did not affect induction of autophagy. In contrast, inhibition of metformin-mediated autophagy prevented PARP cleavage, indicating that metformin-mediated autophagy contributes to metformin-induced apoptosis. This link between autophagy and apoptosis has been previously proposed for targeting melanoma cells in response to cytosolic delivery of dsRNA.31,32

To the best of our knowledge, this is the first demonstration that metformin through concomitant regulation of autophagy and apoptosis favors elimination of cancer cells.

Beclin1 siRNAs were not able to block metformin-induced autophagy and cell death (Supplementary Figure S4), suggesting that metformin induced beclin1-independent autophagy as previously described.33–35 Several recent reports described the central role of the complexes, Beclin1/anti-apoptotic members of the Bcl-2 family in the cross-talk between autophagy and apoptosis.30 The fact that suppression of beclin1 did not modify autophagy and cell death suggested that this protein was not involved in the mechanism of cross-talk. Future studies in our lab will address the mechanism(s) through which metformin signaling induces a specific death in melanoma cells.

Finally, we have evaluated the anti-melanoma activity of metformin in a mouse model of melanoma xenograft (Figure 6). Importantly, we found that short-term administration (3 weeks) of metformin dramatically reduces the development of melanoma tumors in mice. In addition, metformin induces no apparent toxicity, as the body weight (data not shown) and overall appearance of mice given a metformin regimen were not different from those of controls. To determine whether the molecular events observed in cell lines were also found in mice, we performed immunohistochemical staining and TUNEL assay of mouse tumor sections. Experiments showed an increase in LC3 puncta corresponding to autophagosomes and an increase in TUNEL-positive cells in the tumors of metformin-treated mice. These results suggest that in vivo metformin induced autophagy and apoptosis. To circumvent partial immunity deficiency found in nude mice, we also performed the same experiment in a syngeneic model using allograft of B16 melanoma cells in C57Bl6 mice. We have previously checked that metformin...
decreased viability of melanoma B16 cells in vitro (data not shown). Also in this model, metformin diminished the volume and weight of tumor of treated mice, confirming the antineoplastic effect of this compound in vivo.

In summary, we demonstrate for the first time that metformin inhibits melanoma tumor growth through an autophagy-dependent mechanism. This finding brings new clues to the understanding of metformin action in melanoma cell death. Finally, taking into account the drastic effect of metformin on melanoma cell growth and survival in mice, it might be worth evaluating metformin treatment in patients suffering from metastatic melanoma.

Materials and Methods

Reagents and antibodies. Metformin, staurosporine, phenylmethylsulfonyl fluoride, PI, 3-isobutyl-1-methylxanthine, hydrocortisone, insulin, phospho-12 myristate 13-acetate, MCDB 153 medium, sodium fluoride, dimethylacetamide, Hoech3 33258, sodium orthovanadate, 4-(2-aminophenyl)-benzene-sulfonfonyl fluoride, aprotinin and leupeptin were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). The caspase substrates and the caspase inhibitors were from MERCK EuroLab (Fontenay-sous-Bois, France). Trypan blue, Dulbecco's Modified Eagle's Medium (DMEM), penicillin/streptomycin (100 U/ml/50 ng/ml) at 37 °C digested for 1–2 h with collagenase A (0.33 U/ml), dispase (0.85 U/ml) and Dnase I (144 U/ml) with rapid shaking at 37 °C. Large debris were removed by filtration through a 70-μm cell strainer. Viable cells were obtained by Ficoll gradient and penicillin/streptomycin (100 U/ml/50 ng/ml) at 37 °C. Cells were grown in RPMI 1640 (A375, WM9, WM11, WM793, A431, A549, A549-ad, C33, NCI-H292, MDA-MB 435 cells, K562, HeLa) or DMEM (HeLa, A549-AD) according to the manufacturer’s instructions.

Normal human melanocytes were prepared and maintained as described. Different human melanocyte cell lines were purchased from American Type Culture Collection (Molsheim, France). Cells were grown in RPMI 1640 (A375, WM9 and SKmel28) or in DMEM medium (G361 and B16) supplemented with 10% FCS and penicillin/streptomycin (100 U/ml/50 μg/ml) at 37 °C and 5% CO2. Patient melanoma cells were prepared as described. Briefly, biopsy was dissected and digested for 1–2 h with collagenase A (0.33 U/ml), dispase (0.85 U/ml) and Dnase I (144 U/ml) with rapid shaking at 37 °C. Large debris were removed by filtration through a 70-μm cell strainer. Viable cells were obtained by Ficoll gradient centrifugation.

For each experiment, cells were starved with or without 1% SVF in appropriate medium during 14 h before drug stimulation.

Immunofluorescence microscopy. Monolayers prepared for fluorescent microscopy during 14 h before drug stimulation. Metformin, staurosporine, phenylmethylsulfonyl fluoride, PI, 3-isobutyl-1-methylxanthine, hydrocortisone, insulin, phospho-12 myristate 13-acetate, MCDB 153 medium, sodium fluoride, dimethylacetamide, Hoech3 33258, sodium orthovanadate, 4-(2-aminophenyl)-benzene-sulfonfonyl fluoride, aprotinin and leupeptin were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). The caspase substrates and the caspase inhibitors were from MERCK EuroLab (Fontenay-sous-Bois, France). Trypan blue, Dulbecco's Modified Eagle's Medium (DMEM), penicillin/streptomycin (100 U/ml/50 ng/ml) at 37 °C digested for 1–2 h with collagenase A (0.33 U/ml), dispase (0.85 U/ml) and Dnase I (144 U/ml) with rapid shaking at 37 °C. Large debris were removed by filtration through a 70-μm cell strainer. Viable cells were obtained by Ficoll gradient centrifugation.

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Electron microscopy. Electron microscopy experiments were performed as described.

Caspase activity. Caspase activities were performed as described.

Western blot assays. Western blot analyses were performed as described. Quantifications were made using Fuji Film Multi Gauge software.

Flow cytometry analysis. All flow cytometry analyses were performed using the FL2 channels of a FACScan (Becton Dickinson, Cowley, UK) and data were assessed using two-tailed Wilcoxon rank sum test. A value of P < 0.05 was accepted as statistically significant.

Conflict of interest

The authors declare no conflict of interest.

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