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INVESTIGATIVE REPORT

Melanoma Growth and Progression After Ultraviolet A Irradiation: Impact of Lysosomal Exocytosis and Cathepsin Proteases

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Ultraviolet (UV) irradiation is a risk factor for development of malignant melanoma. UVA-induced lysosomal exocytosis and subsequent cell growth enhancement was studied in malignant melanoma cell lines and human skin melanocytes. UVA irradiation caused plasma membrane damage that was rapidly repaired by calcium-dependent lysosomal exocytosis. Lysosomal content was released into the culture medium directly after irradiation and such conditioned media stimulated the growth of non-irradiated cell cultures. By comparing melanocytes and melanoma cells, it was found that only the melanoma cells spontaneously secreted cathepsins into the surrounding medium. Melanoma cells from a primary tumour showed pronounced invasion ability, which was prevented by addition of inhibitors of cathepsins B, D and L. Proliferation was reduced by cathepsin L inhibition in all melanoma cell lines, but did not affect melanocyte growth. In conclusion, UVA-induced release of cathepsins outside cells may be an important factor that promotes melanoma growth and progression. ***Key words: lysosome; cathepsin; UVA; exocytosis; melanocyte; melanoma.***

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Malignant melanoma (MM) arises from pigment-producing melanocytes (MC) in the epidermis. Epidemiological studies have shown that sunlight exposure is an important aetiological factor in cutaneous MM (1). Ultraviolet B (UVB) radiation is thought to be associated with direct DNA damage, whereas the damaging effect of UVA is mediated by oxidative stress-induced alterations, but is less well characterized. MM is the most aggressive form of skin cancer (1). The signalling event that initiates the metastasis process is complex and has not been fully elucidated (2). The initial steps involves invasion through adjacent tissues, which in turn requires degradation of the extracellular matrix (ECM) and the basal membrane. The key players in this process are the matrix metalloproteases (MMPs) and the lysosomal cathepsins, which include the cysteine proteases (e.g. cathepsins B, K and L) and the aspartic cathepsin D (2, 3). Protease overexpression is associated with increased tumour aggressiveness and is often correlated with poor prognosis (4, 5). In addition to the ability to degrade ECM, proteolytically inactive pro-cathepsins have been shown to have mitogenic activities in cancer cells (6, 7).

The lysosomes are the major site of intracellular degradation. Their acidic interior contains more than 50 hydrolytic enzymes (8). The lysosomal proteases cathepsin B, D and L are expressed in most cell types, whereas the others are more cell- and tissue-specific. Cathepsin K, which is highly expressed in osteoclasts and important in bone homeostasis, has also been detected in damaged skin and in tumour stroma (9). Overexpression of cathepsins B, D, L as well as K has been suggested to correlate with poor prognosis in MM patients (9–13).

Interestingly, recent studies have revealed that the function of lysosomes goes far beyond degradation and identified lysosomes as important regulators of both cell death and cell survival. Lysosomal membrane permeabilization releases the lysosomal contents into the cytosol, which has been reported to promote apoptosis (14–16). In addition, the repair mechanism of a torn plasma membrane and cell survival has been shown to involve lysosomal exocytosis (17). Upon plasma membrane damage, Ca^{2+} influx from the extracellular compartment triggers lysosomal fusion with the wounded membrane, forming a re-sealing patch that rescues the cell (17, 18). The lysosomal fusion process is complete in seconds and results in exocytosis of the lysosomal contents (19). In a recent study, we showed that UVA irradiation causes plasma membrane damage in human primary keratinocytes, which is rapidly repaired by Ca^{2+}-dependent lysosomal exocytosis (20). The process was accompanied by release of cathepsin D and acid sphingomyelinase to the cell culture medium. Importantly, the cellular damage induced by UVB, did not involve lysosomal exocytosis and release of cathepsin D to the culture medium.

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Previous studies have shown that proliferation and invasiveness of tumour cells is stimulated by cathepsin secretion. As UV irradiation is important for the development and progression of MM, the aim of the current study is to clarify to what extent UVA, which is the dominant component of sunlight, contributes to these processes. By using matched cell lines derived from primary and metastatic melanomas from the same individual and human MC, we showed that UVA-induced lysosomal exocytosis with release of lysosomal content to the medium results in increased cell proliferation. In addition, both invasion and proliferation was found to be dependent on cathepsin activity.

MATERIALS AND METHODS (see Appendix S1[1])

RESULTS

Expression of cathepsins in melanoma cells

Scrutiny of the gene database Gene Expression Atlas (GEA) revealed several studies of gene expression in human melanomas. Cathepsin B mRNA was highly overexpressed in melanomas compared with melanocytic naevi. In addition, the expression levels of cathepsins D and L were significantly increased, whereas that of cathepsin K was unchanged in melanomas compared with naevi (Fig. 1A). According to the Human Protein Atlas (HPA) database, histological melanoma specimens showed strong/moderate staining for cathepsin B and weak/moderate staining for cathepsin D (Fig. 1B). In a clinical study, cathepsin K staining revealed high expression in 65% of melanomas (21). Data from normal skin is available in the databases, but because MC constitute only a small fraction (approximately 3%) of the cells in the epidermis, we did not consider these data.

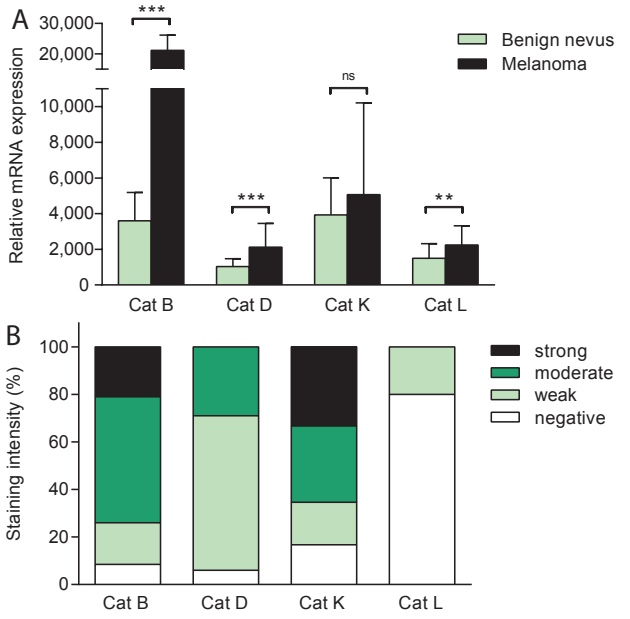

Fig. 1. Cathepsin expression in public databases. (A) Robust Multi-array Average (RMA) normalized expression of cathepsins B, D, K and L in melanomas and benign naevi extracted from the Gene Expression Atlas. Student's *t*-test is used for statistics. (B) Staining intensity of histochemical specimens of melanoma published in the Human Protein Atlas and from Zheng et al. (21). Values are mean ± standard deviation (SD); $^{**}p<0.01$, $^{***}p<0.001$.

Our experimental system consisted of MC and 3 melanoma cell lines established from the same patient: FM55P, from a primary melanoma, and FM55M1 and FM55M2, established from 2 metastases. Using immunoblotting, the intracellular protein expression of cathepsins (B, D, K, L) was investigated, revealing

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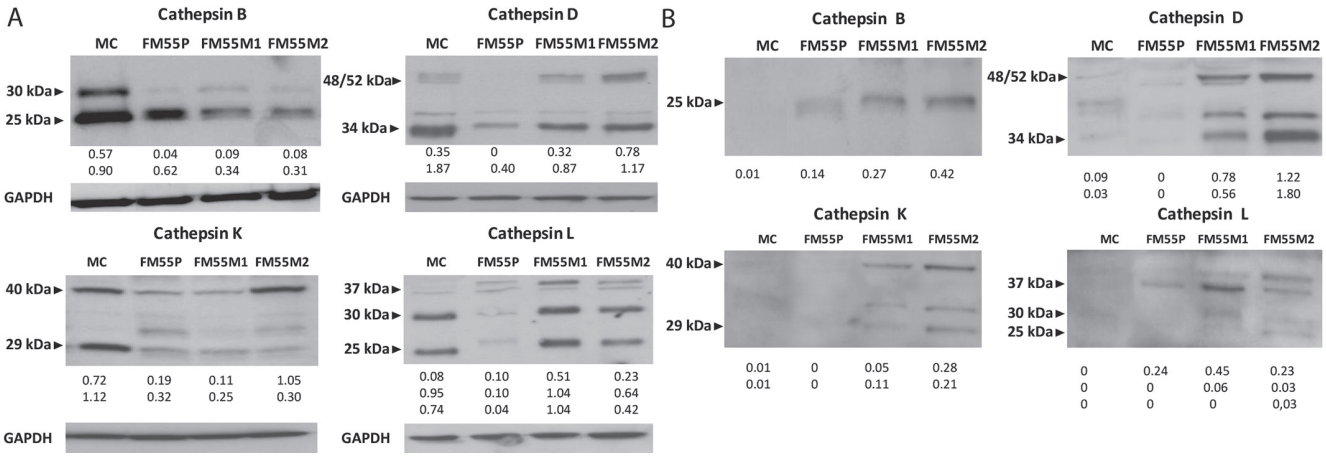

Fig. 2. Expression and secretion of cathepsins. (A) Immunoblot of the protein levels of cathepsins B (pro-form 30 kDa and mature form 25 kDa), cathepsin D (pro-forms 48/52 kDa and mature form 34 kDa), cathepsin K (pro-form 40 kDa and mature form 29 kDa), and cathepsin L (pro-forms 37 and 30 kDa and mature form 25 kDa) using lysates of melanocytes (MC) and the melanoma cell lines FM55P (primary melanoma), FM55M1 and FM55M2 (melanoma metastases). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is used as an internal loading control and optical densities (ODs) of the respective bands correlated to GAPDH are listed below the blots. (B) Immunoblot (1 representative blot out of 3) of spontaneously secreted cathepsins B, D, K and L from concentrated cell culture medium. All received from the same number of cells.

that all these proteins were expressed in all investigated cells (Fig. 2A). MC contained relatively high levels of these cathepsins compared with the melanoma cells. In FM55P cells, the protein levels of cathepsins B, D, K and L were lower than in MC. FM55M1 and FM55M2 showed higher levels of cathepsins D and L than FM55P, but similar or lower levels of cathepsins B and K. Because the invasive potential could depend on the ability to secrete proteases, we analysed the spontaneous secretion of cathepsins by collecting cell culture medium after 24 h of culturing. The MC secretion was below detection limits compared with the malignant cultures (Fig. 2B). FM55P secreted lower levels than the metastatic cell lines, which showed clear secretion of all studied cathepsins.

Lysosomal exocytosis in response to UVA exposure

To analyse lysosomal exocytosis, cells were irradiated in phosphate buffer and unfixed cells were stained immediately with an antibody directed to the intralysosomal NH_2-terminal domain of LAMP-1. Essentially, no LAMP-1 was detected on the plasma membrane of control cells. However, following UVA, LAMP-1 was detected on and in close proximity to the plasma membrane of the cells (Fig. 3A). In accordance with previous findings (20), no increased LAMP-1 staining in the plasma membrane was found after UVB (data not shown). To further verify Ca^{2+}-dependent lysosomal exocytosis, cells were exposed to UVA in PBS with or without Ca^{2+}. In the presence of Ca^{2+} the plasma membrane would be repaired, while Ca^{2+}-free conditions would render cells unable to repair the plasma membrane. Plasma membrane integrity was analysed by addition of the membrane-impermeable dye PI (propidium iodide). After fixation and mounting in DAPI (4',6-diamidino-2-phenylindole), images were captured using a fluorescence microscope. Fig. 3B presents results from FM55P cells showing that irradiation resulted in plasma membrane damage. The damage was rapidly re-sealed in Ca^{2+}-containing buffer, but >80% of cells showed PI uptake when Ca^{2+} was omitted. Quantification of PI-positive cells from all cell lines, showed that a higher proportion of cells were PI-positive in MC and FM55P cells compared with FM55M1 and FM55M2 following UVA exposure (Fig. 3C). UVB did not show any significant increase in PI staining or in Ca^{2+}-containing or Ca^{2+}-free buffer (data not shown), which is in accordance with our previous findings in keratinocytes (20). In addition, we collected PBS from UVA-exposed cell cultures 5 min following irradiation and analysed these samples for cathepsin B, as a marker of lysosomal release, by ELISA. We found significantly increased amounts of cathepsin B released from the FM55P cell line following UVA compared with non-irradiated control cells (Fig. 3D).

Effect of cathepsins on melanoma invasiveness and proliferation

Next, we compared the invasion potential of MC and the melanoma cell lines, using basement membrane extract (BME)-coated membranes. As presented in Fig. 4A, FM55P exhibited high invasive potential resulting in penetration of the membrane by 40% of the cells. In contrast, MC and the FM55M1 and FM55M2 cell lines were not prone to invade through the BME-coating. To evaluate the impact of lysosomal proteases, inhibitors of cathepsins B, D, K and L were added. The inhibition of cathepsin B resulted in a marked reduction in the invasion of FM55P cells, and the inhibition of cathepsins D and L reduced the level of invasion by half compared with the control. Thus, FM55P cells, derived from a primary melanoma, were dependent on lyso-

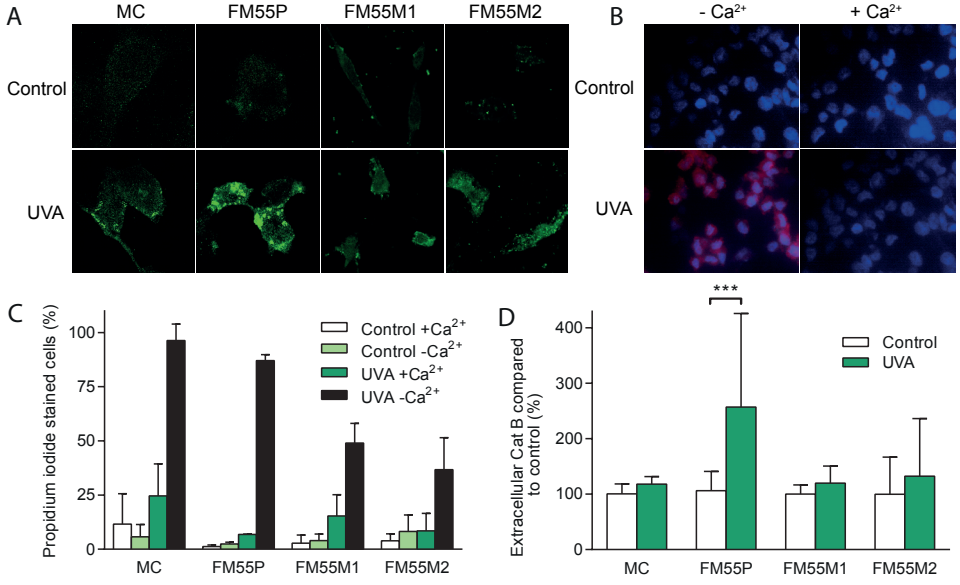

Fig. 3. Ultraviolet A (UVA)-induced lysosomal exocytosis. Melanocytes (MC) and the melanoma cell lines FM55P (primary melanoma), FM55M1 and FM55M2 (melanoma metastases) were exposed to UVA (10 J/cm^2) radiation. (A) Lysosomal associated membrane protein-1 (LAMP-1) localization in unfixed cells immediately after UVA exposure. The antibody used detects the luminal part of LAMP-1. (B) Merged images of FM55P cells showing DAPI (*blue*; all nuclei) and propidium iodide (PI) staining directly following irradiation. PI was added to mark cells that failed to re-seal their plasma membranes. (C) Quantification of cells stained with DAPI and PI, as described in (B). (D) Analysis of cathepsin B released to the culture media after UVA irradiation using ELISA. Student's *t*-test is used for statistics.

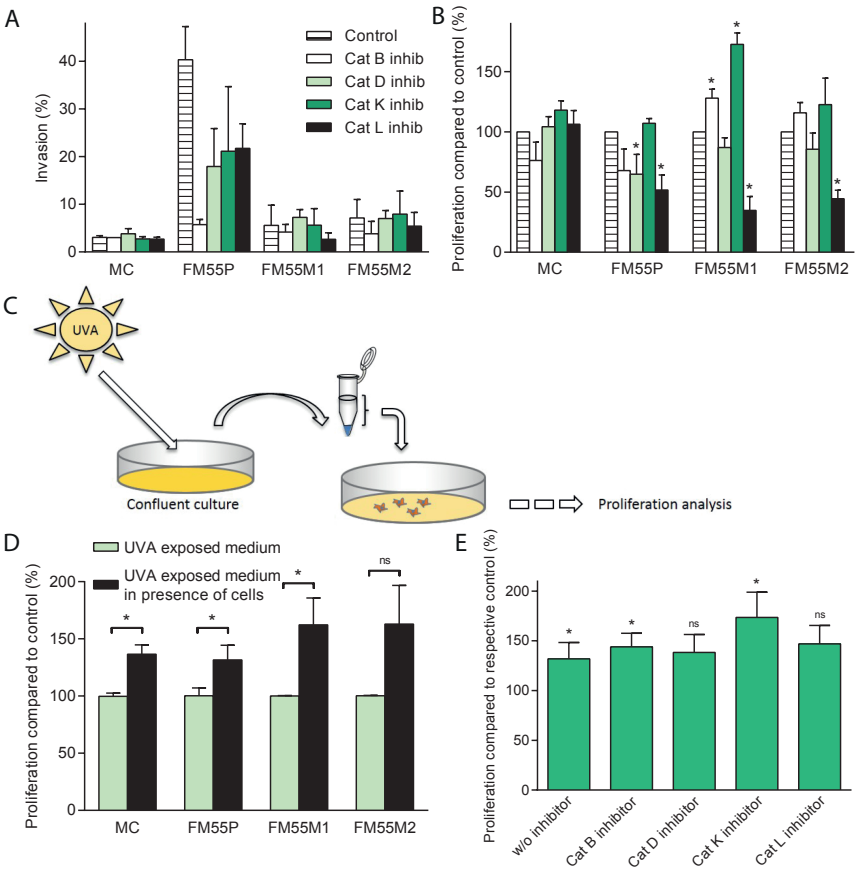

Fig. 4. Effect of cathepsin inhibitors on invasion and proliferation. Melanocytes (MC) and the melanoma cell lines FM55P (primary melanoma), FM55M1 and FM55M2 (melanoma metastases) were treated with inhibitors of cathepsin B, cathepsin L, cathepsin K and cathepsin D. (A) Cell invasion experiments using Cultrex BME. The results are presented as the percentage of invading cells relative to the total cell population (mean ± SEM, $n = 6$). (B) Estimated cell growth expressed as a percentage of the proliferation of the corresponding untreated control. The samples were run in triplicate and each experiment was repeated 4 times ($*p < 0.05$). ANOVA is used for statistics (C) Schematic illustration of the experimental system used in (D) and (E). Following UVA irradiation of confluent cultures, the medium was immediately collected and added to sparsely seeded cells. (D) Cell proliferation in conditioned media. In controls medium was irradiated in the absence of cells. (E) FM55P cultured in UVA-irradiated medium with addition of inhibitors against cathepsins B, L, K and D. The samples were run in triplicate (mean ± SEM, $n = 4$, $*p < 0.05$). D and E: Student's *t*-test is used for statistics.

somal proteases for invasion. No apparent difference in invasion was observed between the untreated and cathepsin-inhibited MC, FM55M1 and FM55M2. No increased apoptosis was detected in cultures exposed to cathepsin inhibitors.

To determine whether cathepsins affect cell proliferation, inhibitors of the respective protease were added. Melanocyte proliferation was unaffected by the inhibition of all investigated cathepsins compared with the untreated control (Fig. 4B). In contrast, the inhibition of cathepsin D resulted in diminished proliferation in FM55P. The most prominent effect on proliferation was, however, observed for the inhibition of cathepsin L, which significantly decreased cell proliferation in all melanoma cell lines. Unexpectedly, the inhibition of cathepsins B and K both resulted in increased proliferation for the metastatic FM55M1 melanoma cells.

Finally, conditioned cell culture media from UVA-irradiated cultures was added to cultures of the respective cell type (Fig. 4C). In MC there was a 40% increase in cell growth for the cultures receiving medium from irradiated cells compared with controls. Likewise, for FM55P, FM55M1 and FM55M2 cells, significant increase in cell growth was observed in cultures grown in medium from irradiated cells compared with cultures receiving medium that had been irradiated in the absence of cells (Fig. 4D). Furthermore, in FM55P we tested whether the addition of inhibitors of cathepsins could alter the observed proliferation pattern. For cathepsins B and K proliferation was increased regardless of whether these cathepsins were inhibited. In contrast, the inhibition of cathepsins D and L reduced the proliferation to a level that did not significantly differ from that of cultures that had received irradiated medium from cell-free cultures, indicating that these cathepsins have a stimulatory growth function (Fig. 4E).

DISCUSSION

In this report we show that UVA causes plasma membrane damage that triggers lysosomal exocytosis and release of lysosomal content outside the cells. Conditioned media from irradiated cells stimulated the growth of un-irradiated cultures, indicating that substances including cathepsins promote proliferation. In separate experiments we found that inhibition of cathepsins D and L reduced proliferation in melanoma cell lines, but not in MC. The spontaneous secretion of cathepsins from melanoma cells was higher than from MC. Taken together, the results suggest that cathepsins affect cell growth and that UVA irradiation with ensuing lysosomal exocytosis is a factor to consider in the progression of a MM.

Lysosomal exocytosis was investigated by analysing the exposure of the luminal domain of LAMP-1 on the

outer leaflet of the plasma membrane. LAMP-1 immunoreactivity was also detected in vacuolar structures in close proximity to the plasma membrane. Such staining is most likely a result of the endocytosis process that restores the plasma membrane after exocytosis (19, 22). Thus, UVA causes plasma membrane damage that trigger cells to exocytose their lysosomes, with subsequent release of lysosomal proteases to the extracellular space. Similar results were recently obtained in human keratinocytes in monoculture and in reconstructed skin exposed to UVA (20, 23). Of interest, Liu et al. (24) found that if the spontaneous release of proteases from lysosomes was prevented by the inhibition of exocytosis, the invasiveness of glioma cells was reduced. We noted increased growth rate when conditioned media from irradiated cultures was added to non-irradiated cell cultures. Due to the fact that the medium was collected directly after irradiation, and the stimulatory effect on proliferation of primary melanoma cells was reduced by the addition of inhibitors of cathepsins D and L, we conclude that lysosomal exocytosis and presence of cathepsins plays a role in the observed effect.

Malignant cells undergo changes in their lysosomal function (25). Here, we found that, in contrast to MC, the melanoma cell lines spontaneously secreted cathepsins into the surrounding medium. The FM55P cells, established from a primary melanoma, showed the highest invasion potential, which was dependent on cathepsin activity. In contrast, the cell lines from metastases, FM55M1 and FM55M2, had a low invasive ability even though their levels of cathepsin secretion were higher than that of the FM55P cells. Thus, the spontaneously secreted amount of cathepsins did not correlate with the invasive capacity. This result indicates that secretion of other proteases, as well as other mechanisms besides secretion, might facilitate invasion. Recent findings have suggested an alternative mechanism of extracellular matrix degradation through the uptake of collagen via the collagen receptors uPARAP/Endo180, which is followed by intralysosomal degradation (26). The gene and protein databases GEA and HPA, respectively, identified high expression of cathepsin B in MMs. Cathepsin B had the highest impact of invasion of FM55P cells. In accordance with our findings, previous investigators have also found cathepsin B to be important in invasion during melanoma progression, both by analysing cellular matrix penetration with Matrigel invasion assays (27) and by inhibiting cathepsin B, which significantly impaired cell invasiveness and metastatic potential in mouse models (13). Cathepsin D has been suggested to be associated with melanoma development (10). FM55M1 and FM55M2 also secreted the pro-form of cathepsin D, which has been shown to stimulate proliferation through a mechanism independent of proteolytic activity (6, 7, 28). In our model, we cannot exclude the growth-stimulating ability of pro-cathepsin D because this function is most likely unaffected by the inhibitor. Cathepsin L was found to be a prominent stimulating factor of proliferation in all melanoma cells studied and in accordance with previous findings invasion was also promoted (29). Cathepsin K has been found to be overexpressed in several MMs (9, 21), but was not confirmed in the cell lines selected here. Moreover, Quintanilla-Dieck et al. (9) reported that only the proform of cathepsin K is present in MC, while we also detected the active form. Importantly, melanocyte growth is insensitive to cathepsin inhibitors. Taken together, these findings indicate that lysosomal cancer associated alterations promote cell growth that depends on cathepsin activity.

In conclusion, this study presents evidence that MM is associated with altered lysosomal function. Furthermore, the UVA-induced exocytosis of lysosomes results in the release of cathepsins outside the cell. Given that cathepsins have been implicated as important mediators of tumour progression, cell invasion, and as ligands that stimulate proliferation, it is reasonable to assume that a MM *in situ* might be influenced by UV radiation at an early stage of the process, which could trigger progression of the tumour.

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