Cathepsin K in Melanoma Invasion

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Cathepsin K (catK) is a lysosomal cysteine protease with strong collagenolytic activity that mediates bone resorption in osteoclasts. Recently, catK expression has been reported in skin and lung fibroblasts, which suggests a role in maintaining homeostasis of the extracellular matrix outside of bone. Matrix degradation is a pivotal step in tumor invasion and metastasis. As other proteases, in particular matrix metalloproteinases and some cathepsins, but not catK, have been described to mediate melanoma invasion, we studied catK in melanoma. Immunostaining revealed strong catK expression in most primary melanomas and all cutaneous melanoma metastases. Melanocytic nevi also demonstrated catK expression, but it was less intense than in melanomas. Melanoma lines express both the pro- and the active form of catK and internalize extracellular collagen into lysosomes. Inhibition of catK greatly reduced melanoma cell invasion through Matrigel basement membrane matrix and increased detection of internalized collagen. We suggest that catK may play an important role in melanoma invasion and metastasis by mediating intracellular degradation of matrix proteins after phagocytosis. Clinical use of catK inhibitors, a class of medication currently in clinical trials for the treatment of osteoporosis, may be a promising avenue for the treatment of melanoma.

Journal of Investigative Dermatology (2008) **128,** 2281–2288; doi:10.1038/jid.2008.63; published online 27 March 2008

INTRODUCTION

Cathepsin K (catK) is a cysteine protease with strong collagenolytic and elastolytic activity involved in extracellular matrix (ECM) turnover that was first characterized as an important mediator of bone resorption by osteoclasts (Drake *et al.*, 1996; Everts *et al.*, 1996; Garnero *et al.*, 1996; Chapman *et al.*, 1997; Yamaza *et al.*, 1998; Xia *et al.*, 1999; Goto *et al.*, 2003). In osteoclasts, catK has been localized to lysosomes, where the inactive precursor pro-catK is activated by auto-proteolysis. It is secreted into the bone resorption lacunae, where it exerts its resorptive activities at a pH optimum of 5.5 (Bossard *et al.*, 1996; McQueney *et al.*, 1997; Dodds *et al.*, 2001; Rieman *et al.*, 2001). CatK has a uniquely high potency to degrade a wide range of collagens, as it is able to cleave at multiple sites within the triple helix of collagens type I and III as well as at extra-helical regions (Garnero *et al.*, 1996). Other proteases are more limited in their proteolytic activity on collagen. For example, matrix metalloproteinases (MMPs) cleave triple helical collagen only at specific single sites, and other cysteine proteases (cathepsins) cleave collagen only at the extra-helical regions. CatK upregulation in osteoclasts is mainly mediated by binding of osteoblast-derived RANKL (receptor activator of nuclear factor-κB (RANK) ligand) to its receptor RANK (Troen, 2006).

While a role of catK was long thought to be limited to bone resorption, it has recently been implicated to be also involved in the turnover of extracellular matrix proteins in other organs. These include the lung, where catK deficiency has been shown to predispose catK-knockout mice to bleomycin-induced lung fibrosis (Bühling *et al.*, 2004), and the skin, where we have found it not to be expressed in normal skin, but upregulated in skin fibroblasts during scar formation, suggesting that its proteolytic activities counteract dermal fibrosing processes in the skin (Rünger *et al.*, 2007).

In one of our surgical scar specimens, we observed catK staining in the cells of a melanocytic nevus. This prompted us to further investigate the role of catK in melanocyte-derived skin lesions, including malignant melanoma.

It is well known that tumor-cell invasion requires interplay of matrix-degrading proteases, growth factors and adhesion molecules, many of which have been extensively studied in melanoma (Smolle *et al.*, 1996; Labrousse *et al.*, 2004).

The stroma surrounding melanoma is characterized by extensive collagen and elastin proteolysis at the invasive front, carried out by a variety of different proteases. These include several members of the MMP family, such as MMP-1, MMP-2, MMP-9, and MMP-13, some of which have been found to be upregulated in melanoma cells as well as surrounding stromal cells. This has also been found to correlate with the invasive and metastatic behavior of melanoma cells and melanoma prognosis (Hofmann *et al.*, 2000a, 2005). Regarding cysteine

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Abbreviations: BCC, basal cell carcinoma; catK, cathepsin K; ECM, extracellular matrix; LAMP1, lysosome-associated membrane protein 1; MMP, matrix metalloproteinase; RANK, receptor activator of NF-κB; RANKL, RANK ligand; SCC, squamous cell carcinoma

Received 8 May 2007; revised 10 January 2008; accepted 1 February 2008; published online 27 March 2008

proteases, cathepsins B and L have been reported to be upregulated in melanoma and suggested to contribute to cell invasion (Fröhlich *et al.*, 2001; Dennhofer *et al.*, 2003; Stabuc *et al.*, 2006). However, the most potent matrix-degrading protease known, catK, has not been studied in melanoma.

RESULTS

Expression of catK in melanoma

Immunostaining of primary cutaneous melanomas revealed moderate, strong, or very strong cytoplasmic expression of catK in 4/5 melanomas *in situ*, 2/2 thin invasive melanomas (Clark level II), and 4/5 thick invasive melanomas (Clark level IV) (Figure 1a). One melanoma *in situ* showed only focal catK expression and one thick and ulcerated melanoma only weak catK expression. Strong staining was observed both in the dermal, as well as in the epidermal, compartment. Two of the invasive melanomas showed declining catK expression in the dermis with increasing depth, whereas the remainder showed uniform strong catK staining throughout the tumor (shown in Figure 1a). The keratinocytes of the overlying epidermis were catK-negative, whereas the pagetoid infiltrate of melanoma cells in the epidermis were positive (Figure 1b). Some catK-positive peritumoral stromal fibroblasts were seen in all invasive primary melanomas except one. All cutaneous melanoma metastases ($n=6$) were strongly and evenly positive for catK (Figure 1c).

Western blot analysis of cultured MM-AN, LIBR, and MeWo melanoma cell lines demonstrated expression of both pro-catK (40 kDa) and active catK (25 kDa) in all melanoma cell lines, whereas cultured primary melanocytes expressed only pro-catK (Figure 1d).

Expression of catK in non-melanoma skin cancer

In contrast to melanomas, basal cell carcinomas (BCCs; $n=3$) and squamous cell carcinomas (SCCs; $n=4$) showed no (1/3 BCCs, 3/4 SCCs) or weak focal expression of catK within tumor cells (Figure 2a–d). However, there was strong expression in the fibroblasts of the peritumoral stroma, which was much more intense than in the peritumoral stroma surrounding some melanomas.

Expression of catK in benign melanocytic nevi

Different types of melanocytic nevi, including compound nevi, intradermal nevi, nevi with features of congenital nevi, dysplastic nevi, and Spitz nevi, demonstrated variable catK staining intensity (Figure 2e–h), ranging from absent through moderate, mostly weaker than the melanomas and melanoma metastases (Table 1). In all but one of the dermal nevi, catK staining was observed not only in the nevus cells, but also in melanocytes in the overlying epidermis (Figure 2f). The one exception to this was a dermal nevus that was completely catK-negative.

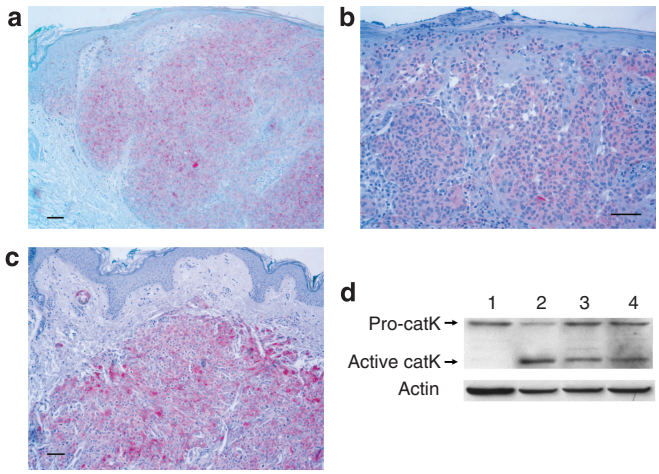

Figure 1. Malignant melanoma cells express catK. Immunostaining of primary cutaneous melanomas for catK shows strong staining in both the dermal and epidermal compartments (**a**, **b**). A cutaneous melanoma metastasis is strongly positive for catK as well (**c**). Western blot demonstrates that cultured melanoma cell lines (MM-AN, LIBR, and MeWo, lanes 2–4, respectively) express both pro-catK (37 kDa) and active catK (25 kDa). Bars = 100 μm. (**d**). Cultured primary melanocytes (lane 1) express pro-catK but not active catK.

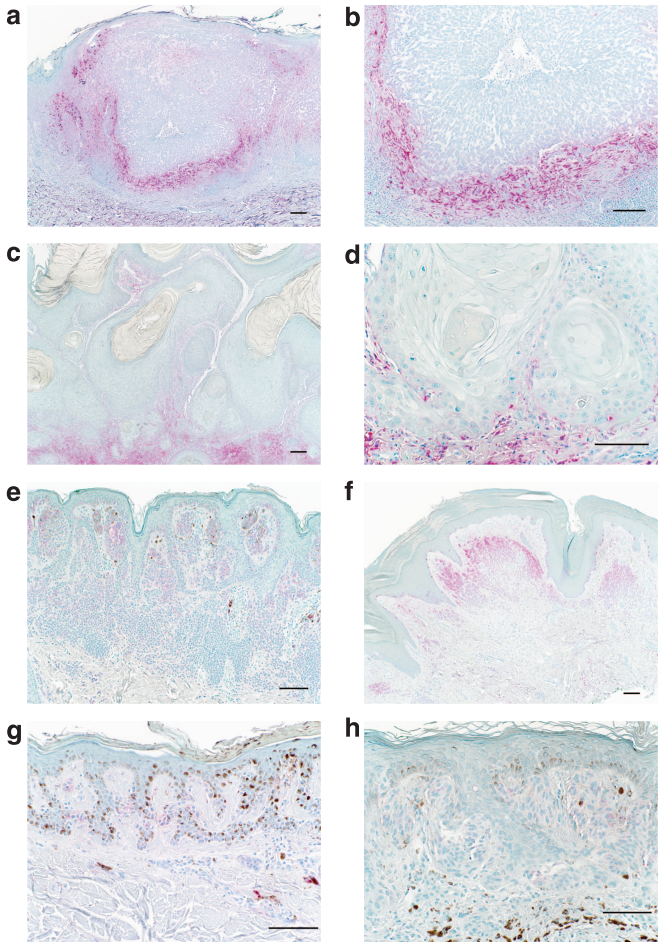

Figure 2. CatK expression in BCC, SCC, and different types of melanocytic nevi. Immunostaining of a BCC (**a** and **b**) shows no catK staining within the tumor cells, but strong catK expression in the peritumoral stroma fibroblasts. Similarly, a SCC (**c** and **d**) shows only weak expression within tumor cells, but very strong expression in peritumoral stroma. There is weak-to-moderate staining in a compound melanocytic nevus (**e**), an intradermal melanocytic nevus (**f**), a dysplastic nevus with mild atypia (**g**), and a Spitz nevus (**h**). Both the compound and the intradermal nevus demonstrate declining catK staining with increasing depth (**e** and **f**). Single melanocytes overlying the intradermal nevus are also catK-positive (**f**). Bars = 100 μm.

Table 1. CatK expression in melanocytic nevi

Type of nevus	CatK staining intensity[1]	Comments on staining pattern
CMN	+	Focal staining, in junctional compartment only
CMN	+	Focal staining, in junctional compartment only
CMN, CNF	+	Focal staining, in junctional compartment only
CMN, CNF	+	Focal staining, in junctional compartment only
CMN, CNF	+	Staining in dermal and epidermal compartments
CMN, CNF	+	Staining in dermal and epidermal compartments
CMN, CNF	++	Staining in dermal and epidermal compartments
CMN, CNF	++	Staining in dermal and epidermal compartments
IDMN	–	
IDMN	+	
IDMN	+	
IDMN, CNF	–	
IDMN, CNF	+	
IDMN, CNF	++	
JDN[2]	+	Focal staining
JDN[3]	+	Focal staining
JDN[3]	+	Focal staining
CDN[2]	–	
CDN[2]	+	Focal, staining in epidermal compartment only
CDN[2]	+	Staining in epidermal compartment only
CDN[2]	++	Staining in epidermal compartment only
JSN[4]	+	
CSN	–	
CSN	+	Staining in epidermal compartment only
IDSN	+	

CatK, cathepsin K; CDN, compound dysplastic nevus; CMN, compound melanocytic nevus; CNF, with histopathological features of congenital melanocytic nevus; CSN, compound Spitz nevus; IDMN, intradermal melanocytic nevus; IDSN, intradermal Spitz nevus; JDN, junctional dysplastic nevus; JSN, junctional Spitz nevus.
[1]–, no staining; +, weak staining; ++, moderate staining; +++, strong staining; ++++, very strong staining.
[2]Mild atypia.
[3]Moderate atypia.
[4]None of the Spitz nevi were atypical Spitz nevi.

CatK expression in melanoma is not regulated by the RANK/RANKL pathway

Western blot analysis demonstrated the expression of RANK and RANKL by three different melanoma cells lines (Figure 3a). The expression levels of RANK and RANKL in these lines varied and were inversely related, with melanoma lines with the highest RANK expression (MM-AN) showing the lowest RANKL expression and vice versa (LIBR and MeWo). However, treatment with RANKL did not increase catK expression in these three melanoma lines (Figure 3b–d), suggesting that activation of the RANK/RANKL pathway does not regulate catK expression in melanoma cells.

Inhibition of catK inhibits melanoma invasion

With MeWo and MMAN melanoma cells plated onto the Matrigel-coated membranes, treatment with the catK inhibitor greatly reduced the number of invading cells (Figure 4). When placed onto uncoated control membranes and counted after 18 (MeWo) or 20 hours (MMAN), the mean number of diluent-treated cells migrating through the membrane was 274 with MeWo cells and 159 with MM-AN cells. The number of catK inhibitor-treated (any concentration) melanoma cells migrating through the uncoated membrane was not statistically different from the number of diluent-treated cells. This indicates that the inhibitor was not toxic and did not affect the ability of the cells to migrate (as opposed to their ability to invade through Matrigel). A mean number of 156 MeWo cells or 80 MMAN cells invaded through the Matrigel with diluent treatment, indicating an invasion index of 56.9 and 50.4% for the two cell lines. When estimating a plating efficiency of approximately 50, 0.6% of diluent-treated MeWo cells and 0.3% of diluent-treated MM-AN cells had invaded through the Matrigel at the indicated time points. An invasion index of 0.11 with MeWo cells and of 0.02 with MMAN cells with the highest concentration of the catK inhibitor indicates a reduction of invasion by 89 and 98%, respectively. A dose response was observed, as lower doses of the catK inhibitor demonstrated less reduction of invasion. LIBR cells did not migrate through the uncoated membrane of the invasion chamber membrane and therefore could not be used for these invasion studies.

Melanoma cells internalize collagen IV

Twenty-four hours after adding Oregon green-labeled collagen IV to melanoma cells, internalized collagen was detectable in 14.3% of MeWo and 17.7% of MM-AN cells (Figure 5a and c). The internalized collagen colocalized with LAMP1 (lysosome-associated membrane protein 1), which demonstrates that internalized collagen is located in lysosomes (=late endosomes). Observations over time after adding labeled collagen revealed that at the 1-, 3-, and 9-hour time points most internalized collagen is found close to the cell borders and does not colocalize with LAMP1 (Figure 5b, left panel). This is consistent with localization of internalized collagen in early (LAMP1-negative) endosomes at these early time points. At the later time points (16, 21, 24, and 26 hours after adding collagen IV), most of the internalized collagen was found all over the cytoplasm and colocalized with LAMP1, demonstrating its localization in lysosomes (Figure 5b, middle panel). At the latest time point (26 hours), some cells were observed in which internalized collagen was restricted to one small perinuclear, LAMP1-positive compartment (Figure 5b, right panel). With inhibition of catK, the fraction of cells with detectable internalized

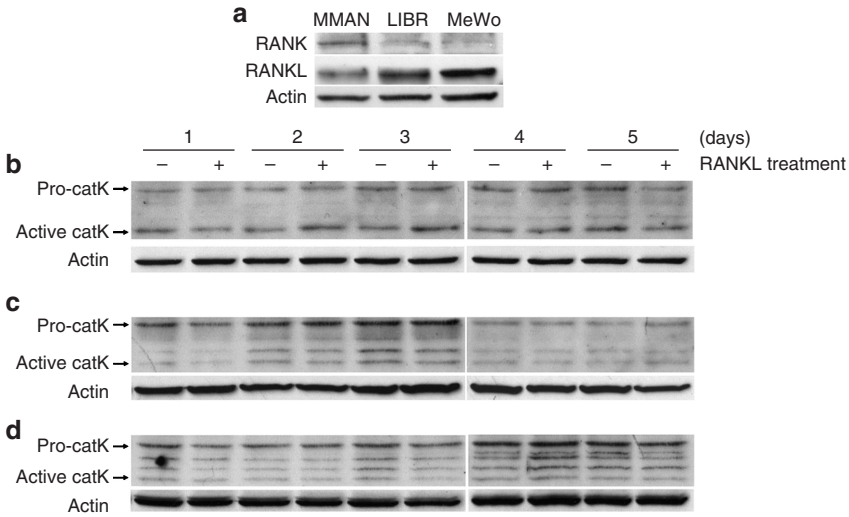

Figure 3. Treatment with RANKL does not induce catK expression in melanoma cells. Western blot demonstrates that cultured melanoma cell lines MM-AN, LIBR, and MeWo express both RANK and RANKL to varying degrees (**a**). However, treatment of cultured melanoma cells with RANKL did not induce catK expression in either cell line, as compared with diluent-treated cells (MM-AN (**b**); LIBR (**c**); MeWo (**d**)). RANKL (80 ng ml^{-1}; R&D Systems) or diluent (complete medium) was added to non-confluent, exponentially growing cells once and protein was harvested for immunoblotting on five consecutive days.

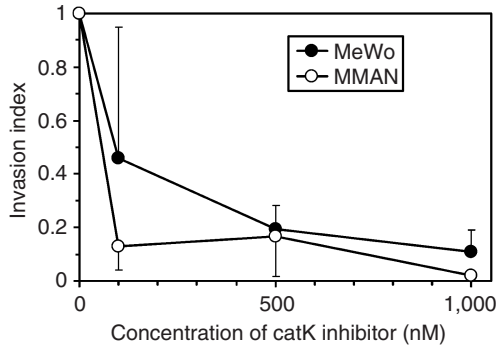

Figure 4. Inhibition of catK dose dependently inhibits invasion of MeWo and MMAN melanoma cells through a reconstituted basement membrane. Shown are means ± SD of triplicate samples 18 (MeWo) or 20 hours (MM-AN) after cell plating.

collagen IV increased 2.1- and 2.6-fold for MeWo and MM-AN cells, respectively (Figure 5c; $P = 0.03$). This suggests that rapid degradation of internalized collagen by catK limits detectability of internalized collagen.

DISCUSSION

Here we describe, to our knowledge for the first time, expression of catK in malignant melanoma and its cutaneous metastases, both *in-vivo* and *in-vitro*. As catK is the most potent collagenase known, this suggests that it may play an important role in melanoma invasion and metastasis. This is further supported by our findings that inhibition of catK greatly reduces invasion of two different melanoma cell lines in an *in-vitro* invasion assay.

Mechanisms that enable tumor cells in general to invade into adjacent tissue and, during metastasis, into blood and lymph vessels and into distant tissues, have been subject of intense research. One crucial aspect of tumor invasion and metastasis is degradation of the extracellular matrix. Much research has focused on MMPs as mediators of this process (Curran and Murray, 1999; Stetler-Stevenson, 2001; Yoon *et al.*, 2003; Deryugina and Quigley, 2006). For melanoma in particular, MMPs have been shown to play a role in tumor invasiveness and metastatic behavior *in vivo* and *in vitro*, including mouse models, and to correlate with tumor stages and clinical prognosis in patients (Ray and Stetler-Stevenson, 1995; Itoh *et al.*, 1998, 1999; Schultz *et al.*, 1988; Vaisanen *et al.*, 1998; Hofmann *et al.*, 2000a, b, 2003, 2005; Nikkola *et al.*, 2002, 2005; Iida *et al.*, 2004). Treatment of melanoma cells with an MMP inhibitor was shown to reduce melanoma cell invasion through Matrigel, but only by 55% (Durko *et al.*, 1997), which is less than the 87 and 98% reduction we observed with treatment of MeWo and MMAN melanoma cells with a catK inhibitor. Given all these data, MMPs have been considered promising targets for anticancer therapies. However, clinical trials using several generations of MMP inhibitors failed to slow tumor progression (Coussens *et al.*, 2002; Overall and Kleifeld, 2006b). This may indicate that MMPs, although contributory, are not the most important players in tumor-mediated extracellular matrix degradation.

MMPs degrade ECM proteins in the extracellular environment. Recently, however, the intracellular degradation of ECM proteins has been suggested to be more important for tumor invasion (Overall and Kleifeld, 2006a). Fibroblasts are known to internalize extracellular collagen via phagocytosis after binding of collagen to collagen receptors (for example, α2β1 integrin) (Lee *et al.*, 1996). Given that melanoma cells express a variety of collagen receptors (Kramer and Marks, 1989), it is not surprising that melanoma cells, as we show here, are as well capable of internalizing extracellular collagen via endocytosis into lysosomes. The high expression of the lysosomal protease catK in melanoma cells suggests

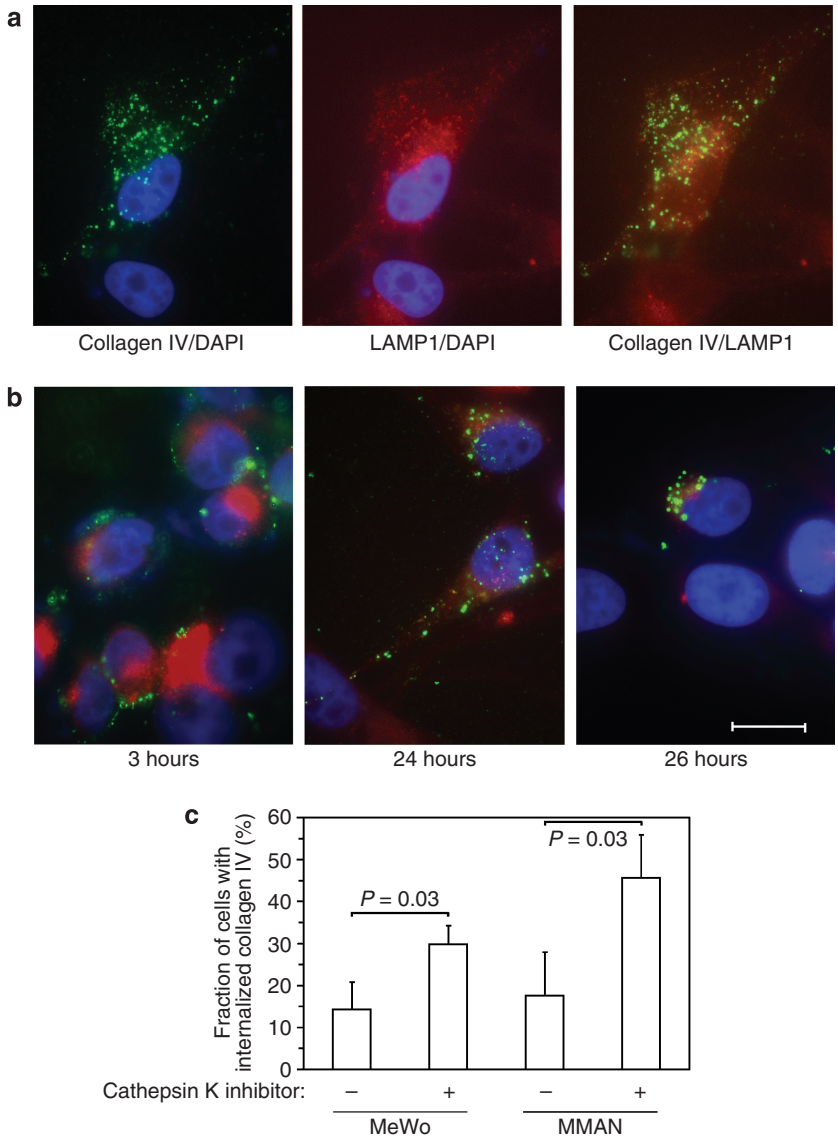

Figure 5. Melanoma cells internalize collagen IV. Twenty-four hours after adding Oregon green-labeled collagen IV, many MeWo melanoma cells demonstrate intracellular collagen IV (**a**). Colocalization with the lysosomal protein LAMP1 demonstrates that internalized collagen IV is located in lysosomes. Green, collagen IV; blue, DNA (4′,6-diamidino-2-phenylindole); and red, LAMP1. Time-dependent progression through different stages of endocytosis during collagen IV internalization in MeWo melanoma cells. (**b**) Three hours after adding Oregon green-labeled collagen IV, most of the intracellular collagen IV was located along cell borders and did not colocalize with LAMP1. This is consistent with localization in early endosomes. Within 24 hours, internalized collagen IV is found all over the cytoplasm within LAMP1-positive lysosomes (late endosomes). Within 26 hours, some cells demonstrate concentration of internalized collagen in perinuclear lysosomes. Bar = 10 μm. Inhibition of catK increases detection of collagen IV internalization in MeWo and MM-AN melanoma cells (**c**). Five hours before adding Oregon green-labeled collagen IV, cells were treated with the catK inhibitor Boc-1. Twenty-four hours after adding labeled collagen, internalization of collagen IV was scored in three separate fields (100 cells each). Shown are means ± SD.

that it contributes to intracellular degradation of internalized collagen through its strong and versatile collagenolytic activity. This is further supported by our finding that inhibition of catK increased detection of internalized collagen, presumably because the high levels of catK in melanoma cells rapidly degrade internalized collagen when not inhibited. Therefore, we would like to suggest catK as a promising target for treatment of malignant melanoma. Potent catK inhibitors have already been developed and investigated in clinical trials for the treatment of osteoporosis (Adami *et al.*, 2006). Unlike MMPs, which are produced predominantly by the peritumoral stromal cells, melanoma cells themselves strongly express catK, making catK-mediated ECM degradation independent of the peritumoral stroma. Nevertheless, as we show that most melanomas are characterized also by an upregulation of catK in peritumoral fibroblasts, inhibition of catK would not only target tumor cell-, but also stroma cell-mediated ECM degradation.

In osteoclasts, catK degrades bone collagen not only intracellularly, after internalization into lysosomes, but also

extracellularly after secretion into bone resorption lacunae. The low pH optimum of catK limits its extracellular activity to acidic environments. Given that a tumor microenvironment is often acidic, it is perceivable that secreted catK could also contribute to ECM degradation of tumors. The pH of human melanoma, as measured by electrodes, has been reported to be within the range of 6.4–7.3 (Vaupel *et al.*, 1989), which is likely not low enough for secreted catK to be highly active. However, some melanoma micro-compartments may have an even lower pH, so that a collagenolytic activity of catK in the extracellular space cannot be entirely excluded. The observation that an acidic extracellular pH promotes melanoma metastasis in mice (Rofstad *et al.*, 2006) may be interpreted to support a role of extracellular catK activity in melanoma. However, given catK's intracellular collagenolytic activity in acidic lysosomes, its matrix-degrading capabilities are not limited to an acidic environment.

Osteolysis in bone metastasis, including melanoma bone metastasis, has often been thought to be mediated by a tumor cell-mediated activation of osteoclasts (Hiraga *et al.*, 1995; Guise *et al.*, 2006). However, evidence that melanoma cells can also directly degrade bone has been provided by Sanchez-Sweatman *et al.* (1997) using a mouse melanoma model. Given that melanoma cells strongly express catK themselves, as we show here, it is possible that in melanoma bone metastases, the tumor cells themselves degrade bone, and are not dependent on an activation of osteoclasts. Therefore, treatment with a catK inhibitor might be particularly promising for the treatment of bone metastases of melanoma, as it would inhibit bone degradation not only by osteoclasts, but also by melanoma cells themselves.

Alternatively, instead of using a catK inhibitor to reduce tumor invasion and metastasis, targeting catK-stimulating signals may be another treatment avenue. Recently, inhibition of RANKL, a potent regulator of catK, has been suggested for the treatment of lytic bone lesions in multiple myeloma (Heath *et al.*, 2007). Here, we show that melanoma cells express both RANK and RANKL. However, treatment with RANKL did not induce catK expression in these cells, suggesting that treatment with a RANKL inhibitor may not have the desired effects on catK expression in melanoma.

CatK immunostaining showed clearly different staining patterns for melanomas and non-melanoma skin cancers. Unlike melanomas, SCCs and BCCs do not or only focally express catK. However, they demonstrate prominent catK staining in the peritumoral stroma, the invasion front in particular. It has previously been shown that BCC tumor cells demonstrate low proteolytic activity when compared with adjacent stromal tissue (Schlagenhauff *et al.*, 1992), suggesting that the dense fibrotic stromal tissue in these types of tumors does not constitute an inert barrier to invasion, but rather is an active contributor to tumor growth and invasion. We would like to add that stromal catK expression might contribute, at least in part, to invasion of non-melanoma skin cancers. It is noteworthy that both SCCs and BCCs readily invade bone per continuum, likely to be mediated through activation of osteoclasts at the invasion front. However, when these tumor cells encounter an invasion barrier in which catK cannot be induced, they would fail to invade and produce metastasis. This may explain why BCCs, and to a lesser degree SCCs, are less prone to metastasize than melanoma.

The expression of catK is not limited to malignant melanoma, but is also found in benign melanocytic nevi. One might speculate that catK expression in nevus cells enables them to invade into the dermis, which is part of their natural ''life cycle'' and is unrelated to malignancy. Most catK-expressing nevi showed the strongest catK expression in the epidermis and upper dermis, and declining catK expression with increasing depth. While this was seen in some melanomas, many melanomas did not demonstrate such ''maturation''.

Melanocytes in normal skin were found to not express catK. However, we do not believe that catK expression differentiates melanoblasts/nevus cells from melanocytes, as we found that melanocytes in the epidermis overlying most dermal nevi were catK positive and that melanocytes in culture expressed catK as well. It, therefore, appears that melanocytes have an innate capability to express catK, but need certain stimuli, presumably present in melanocytic nevi and melanomas, to do so. An innate capability of melanocytes to invade, for example, during the natural ''life cycle'' of nevi or during embryonal migration from the neuroectoderm into the epidermis may explain the ease with which melanomas invade and metastasize. It is tempting to speculate that catK is involved.

MATERIALS AND METHODS

Immunostaining

Immunostaining was performed on formalin-fixed, paraffin-embedded sections of six primary cutaneous melanomas, seven cutaneous melanoma metastases, three BCCs, four SCCs, and 25 melanocytic nevi of different types. The melanocytic nevi included eight compound nevi (of which six displayed histopathological features of congenital nevi), six intradermal nevi (of which three had histopathological features of congenital nevi), three junctional dysplastic nevi with mild or moderate atypia, four compound dysplastic nevi with mild atypia, and four Spitz nevi (one junctional, two compound, and one intradermal; none of which were atypical). For catK-staining we used a monoclonal catK antibody (Novocastra, Newcastle upon Tyne, UK) at a dilution of 1:40 for 32 minutes on an automated immunohistochemistry system (Ventana Benchmark LT; Ventana Medical Systems, Tucson, AZ), and counterstained with hematoxylin and a postcounter staining with blueing reagent (Ventana Medical Systems).

Cell culture

Primary human melanocytes were obtained from surgical specimens of neonatal foreskin as described previously (Gilchrest *et al.*, 1984), and cultured in calcium-free minimal essential medium 199 (Gibco/Invitrogen, Grand Island, NY) supplemented with 5% fetal calf serum, insulin, T3, transferring, epidermal growth factor, basic fibroblast growth factor, hydrocortisone, and inositol. The melanoma cell lines MMAN, MeWo, and LIBR were cultured in DMEM (Gibco/BRL, Rockland, MA) supplemented with 10% calf serum, and grown at 37 °C in a humidified 5% CO_2 atmosphere. LIBR is derived from a

primary malignant melanoma; MMAN and MeWo from melanoma metastases.

Western blotting

Western blot analysis was performed employing standard procedures, with proteins harvested from subconfluent exponentially growing cells. Antibodies used were directed against catK (rabbit polyclonal, detects both the pro- and the active form of catK, 1:200 dilution; Abcam, Cambridge, MA), RANK (mouse mAb, 1:170 dilution; R&D Systems, Minneapolis, MN), RANKL (mouse mAb, 1:140 dilution; R&D Systems), and actin for loading control (goat polyclonal, horseradish peroxidase-conjugated, 1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA).

Melanoma cell invasion assay

Melanoma cell lines were treated with the diluent or the lysosomotropic, water-soluble polymer selective catK inhibitor Boc-I (catK inhibitor II; Calbiochem, La Jolla, CA) at concentrations of 0.1, 0.5, and 1 μM for 5 hours (Wang *et al.*, 2002). Complete inhibition of catK has been demonstrated for the 1 μM concentration in synovial fibroblasts. Other cathepsins are much less inhibited by this agent (K_i 90–10,000-fold higher). Fifty thousand cells in serum-free medium were then placed into the inserts of BioCoat Matrigel invasion chambers (Becton Dickinson Biosciences, Bedford, MA) and again treated with the catK inhibitor. In the invasion chamber inserts, an 8-μm pore size polyethylene terephthalate membrane is coated with basement membrane matrix, and cells invade and migrate along a nutrient gradient provided by complete medium (5% fetal bovine serum) in the chamber well. Parallel control experiments were performed with uncoated chamber inserts. After 18 (MeWo) or 20 hours (MMAN), all cells that invaded through the matrix and migrated through the pores of the membrane were counted after methanol fixing and staining with Diff-Quik (Dade Behring, Newark, DE) using a light microscope. The assay was performed in triplicates. Percent invasion was calculated as the ratio of the mean number of cells migrating through the Matrigel-covered membrane and the mean number of cells migrating through the control membrane (not covered by Matrigel). The invasion index was calculated as the ratio between % invasion of the catK inhibitor-treated cells and the % invasion of diluent-treated cells.

Collagen IV internalization and localization experiments

Internalization of collagen IV by melanoma cells was studied as described by Kjøller *et al.* (2004) for fibroblasts. Briefly, cells were seeded onto glass coverslips and grown for 48 hours in complete medium. Thirty minutes prior to adding Oregon green-labeled collagen IV (25 μg ml^{-1}; Molecular Probes-Invitrogen, Carlsbad, CA), cells were washed twice in ice-cold serum-free medium and kept at 4 °C for 30 minutes. For inhibitor studies, cells were preincubated with the selective catK inhibitor Boc-I (1 μM; catK inhibitor II, Calbiochem) or diluent for 5 hours prior to incubation in collagen IV.

Cells were kept at 4 °C for another 2 hours to allow binding of collagen before transfer to 37 °C for various periods of time (1, 3, 9, 16, 21, 24, and 26 hours). For quenching of extracellular, non-internalized collagen, cells were incubated with 0.4% trypan blue (Sigma-Aldrich, St Louis, MO) in saline solution for 5 minutes before washing and fixation. Cells were fixed in 4% paraformaldehyde (10 minutes at room temperature) and permeabilized by immersion in methanol/acetone (1:1) at −20 °C for 1 minute. After blocking with 10% goat serum (Jackson ImmunoResearch, West Grove, PA) in 0.1% nonidet-40/phosphate-buffered saline (30 minutes at room temperature), cells were incubated with mouse anti-LAMP1 (BD Pharmingen, San Jose, CA), 1:250, in 1% BSA/phosphate-buffered saline (16 hours at 4 °C). After washing with 0.1% nonidet-40/phosphate-buffered saline (3 × 10 minutes), cells were incubated with a secondary antibody (1:500; Rhodamine Red-X-conjugated anti-mouse IgG; Jackson ImmunoResearch) for 1 hour at room temperature, washed three time with 0.1% nonidet-40/phosphate-buffered saline, and mounted on glass slides using a 4′,6-diamidino-2-phenylindole-containing embedding medium (Vectorshield; Vector Laboratories, Burlingame, CA). Cells were inspected with an Eclipse E400 fluorescence microscope (Nikon, Melville, NY) and images were acquired with a Spot RT digital camera and Spot Advanced RT software (Diagnostic Instruments, Sterling Heights, MI). Co-staining of internalized collagen with LAMP1 indicates localization in lysosomes/late endosomes, whereas missing co-staining with LAMP1 is consistent with localization in early endosomes, which are LAMP1-negative and usually localize closer to the cell membrane.

All studies have been approved by the authors' Institutional Review Board and the Declaration of Helsinki Principles have been followed. Patient consent was not required because data were collected without patient identifiers.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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