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Secretion of Active Membrane Type 1 Matrix Metalloproteinase (MMP-14) Into Extracellular Space in Microvesicular Exosomes

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ABSTRACT

Membrane type 1 matrix metalloproteinase (MT1-MMP, MMP14) is an efficient extracellular matrix (ECM) degrading enzyme that plays important roles in tissue homeostasis and cell invasion. Like a number of type I membrane proteins, MT1-MMP can be internalized from the cell surface through early and recycling endosomes to late endosomes, and recycled to the plasma membrane. Late endosomes participate in the biogenesis of small (30–100 nm) vesicles, exosomes, which redirect plasma membrane proteins for extracellular secretion. We hypothesized that some of the endosomal MT1-MMP could be directed to exosomes for extracellular release. Using cultured human fibrosarcoma (HT-1080) and melanoma (G361) cells we provide evidence that both the full-length 60 kDa and the proteolytically processed 43 kDa forms of MT1-MMP are secreted in exosomes. The isolated exosomes were identified by their vesicular structure in electron microscopy and by exosomal marker proteins CD9 and tumor susceptibility gene (TSG101). Furthermore, exosomes contained 1-integrin (CD29). The exosomes were able to activate pro-MMP-2 and degrade type 1 collagen and gelatin, suggesting that the exosomal MT1-MMP was functionally active. The targeting of MT1-MMP in exosomes represents a novel mechanism for cancer cells to secrete membrane type metalloproteolytic activity into the extracellular space. J. Cell. Biochem. 105: 1211–1218, 2008.

KEY WORDS: MATRIX METALLOPROTEINASE; MT1-MMP; EXOSOME; CD9; TSG101; 1-INTEGRIN; CD29; CANCER; VESICLE

Matrix metalloproteinases (MMP) are zinc-dependent endopeptidases, which participate in tissue remodeling during physiological and pathological processes including cell migration, matrix remodeling, and tumor invasion. Most MMPs are synthesized and secreted as latent precursor enzymes (pro-MMP), which are processed extracellulary to their active forms. The expression of membrane type matrix metalloproteinases (MT-MMP) is limited to the cell membranes via a glycophosphoinositol anchor or a transmembrane domain [Itoh et al., 1999]. MT1-MMP, which belongs to the latter group, is one of the most critical metalloproteinases that enhance tumor invasion by remodeling the extracellular matrix. MT1-MMP degrades, for example, fibrillar collagens, fibronectin, fibrin, proteogycans, and laminins 1 and 5 [Ohuchi et al., 1997; Hiraoka et al., 1998; Itoh and Seiki, 2006].

MT1-MMP is expressed as an inactive precursor, cleaved intracellulary by furin and delivered to the plasma membrane in the active form [Sato et al., 1996; Lehti et al., 2000; Yana and Weiss, 2000; Osenkowski et al., 2004]. Several processes have developed to control the amount of active MT1-MMP at the plasma membrane. These include autocatalytic processing, homodimerization, ectodomain shedding, and endocytosis [Lehti et al., 1998, 2000; Jiang et al., 2001; Uekita et al., 2001; Osenkowski et al., 2004]. Autocatalytic processing of MT1-MMP results in a 20 kDa soluble fragment and inactive 43 kDa species that remains at the cell membrane [Lehti et al., 1998, 2000; Toth et al., 2002]. In non-autocatalytic shedding, so far unidentified metalloproteases release two soluble forms (50 and 52 kDa) of the ectodomain of MT1-MMP, which retain the catalytic activity [Toth et al., 2002, 2006].

Internalization of MT1-MMP from the cell surface and cycling between plasma membrane and cell endocytic compartments regulate the enzyme availability at the cell surface [Jiang et al., 2001; Uekita et al., 2001; Gálvez et al., 2002; Remacle et al., 2003]. MT1-MMP is internalized in early endosomes through clathrin and dynamin-dependent pathways [Jiang et al., 2001; Uekita et al.,

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2001]. After internalization MT1-MMP is colocalized with the markers of various endocytic compartments, including early endosomes (EEA1 and Eps15), recycling endosomes (Rab4), and late endosomes/lysosomes (LAMP-1) [Remacle et al., 2003]. The colocalization of MT1-MMP with Rab4 suggests that at least part of the internalized MT1-MMP is recycled to cell surface [Remacle et al., 2003].

Late endosomes participate in the biogenesis of small (30–100 nm) vesicles, exosomes, which redirect plasma membrane proteins for extracellular secretion [Thery et al., 2002]. We hypothesized that a fraction of the endosomal MT1-MMP could be directed to exosomes for extracellular release. Consistent with this prediction, a fraction of MT1-MMP from fibrosarcoma was sedimented during ultracentrifugation of the cell culture supernatants. The present study was thus aimed at analyzing the nature, origin, and activity of the pelleted MT1-MMP. We found that both the full length (60 kDa) and the inactive (43 kDa) forms of MT1-MMP were secreted in exosomes. Current results reveal a novel mechanism for the constitutive release of active MT1-MMP from the tumor cells into the extracellular space.

MATERIALS AND METHODS

ANTIBODIES

Rabbit polyclonal antibody (pAb) ab-1 against human MT1-MMP is described in Lehti et al. [1998]. A mouse monoclonal antibody (mAb) to CD9 and 1-integrin were obtained from Immunotools GmbH (Friesoythe, Germany). A monoclonal antibody for TSG101 was obtained from Abcam plc. (Cambridge, UK).

CELLS

The human fibrosarcoma (HT-1080) and melanoma (G361) cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). HT-1080 cells were stably transfected with MT1-MMP as described in Lehti et al. [1998]. The cells were maintained in Modified Eagle's Medium (MEM; Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco), 2 mM L-glutamine (BioWhittaker, Walkersville, MD) and antibiotics (10 U/ml of penicillin and 10 g/ml of streptomycin) at 37°C in a humidified atmosphere containing 5% CO_2.

EXOSOME ISOLATION

The cells (3.2×10^8) were cultured to subconfluency as described above. The cell layers were washed with phosphate buffered saline (PBS) and cultured further in MEM supplemented with FCS (5%) that had been ultracentrifuged overnight at 100,000*g* to exclude bovine exosomes. FCS was omitted from some cultures where indicated. Exosomes were isolated by differential centrifugation as described [Gutwein et al., 2005]. Briefly, the cell culture media were centrifuged for 300*g* for 10 min to remove detached cells. The supernatants were then centrifuged once at 2,000*g* for 15 min. After centrifugation at 10,000*g* for 30 min the supernatants were ultrafiltered through a membrane of 0.22 m pore size (Millipore, Bedford, MA). Finally, exosomes were pelleted at 100,000*g* for 2 h.

SUCROSE DENSITY GRADIENT FRACTIONATION OF EXOSOMES

Pelleted vesicles were suspended in PBS containing the protease inhibitor cocktail (Complete; Roche Diagnostics GmbH, Mannheim, Germany) and loaded on top of a sucrose gradient consisting of layers of 0.25/0.5/0.8/1.16/1.3/2 M sucrose in 50 mM Tris–HCl (pH 7.5) containing Complete [Gutwein et al., 2005]. After centrifugation at 100,000*g* for 2.5 h in Beckman SW41.1 Ti rotor, 12 fractions of 1 ml were collected from the top of the gradients. The fractions were diluted with 10 ml of PBS (pH 7.5) and the vesicles were pelleted by ultracentrifugation.

ISOLATION OF CELL MEMBRANES

G361 cell membranes were isolated as previously described with some modifications [Gelderman et al., 2003]. Briefly, G361 cells were isolated by scraping into 2 ml of ice-cold hypotonic lysis buffer (10 mM Tris, 10 mM NaCl, and 1.5 mM $MgCl_2$, pH 7.4) containing Complete and were incubated further on ice for 5 min. The cell membranes were disrupted by stroking the suspension with a Teflon pestle in a glass homogenizer. After removing the nuclei by centrifugation twice at 1,500*g* for 5 min, the cell membranes were pelleted at 10,000*g* for 30 min. Finally, the protein concentrations of the cell membrane and exosomal samples were determined (Micro BCA™ Protein Assay Kit, Pierce, Rockford, IL).

SDS–PAGE AND IMMUNOBLOTTING ANALYSIS

The proteins of the isolated exosomes were separated by 10% SDS–PAGE and transferred onto nitrocellulose filters (Schleicher & Schuell, Dassell, Germany). After saturating the excess protein binding sites on the membranes with 5% (w/v) non-fat milk powder in Tris-buffered saline (TBS, pH 7.5) for 15 min the filters were incubated overnight at +4°C with the pAb to MT1-MMP or mAb to CD9, TSG101 or 1-integrin in TBS containing 5% non-fat milk. In the controls, the primary antibodies were omitted or replaced with an irrelevant antibody of the same subclass as the specific mAb. After washing with TBS the bound antibodies were visualized using peroxidase-conjugated anti-rabbit or anti-mouse IgG and an enhanced chemiluminescence substrate (ECL).

IMMUNOELECTRON MICROSCOPY (IEM) OF MT1-MMP ON THE ISOLATED EXOSOMES

An aliquot of each fraction of the sucrose density gradient centrifugation was adsorbed on gold-coated copper grids and contrasted with uranyl-oxalate (2%, pH 7.0) for 1 min for transmission electron microscopy (TEM) analysis. For IEM, the exosomes were adsorbed on gold-coated copper grids and fixed with 4% paraformaldehyde for 10 minutes. After blocking with 1% fish skin gelatin and 1% bovine serum albumin (BSA) the samples were immunolabeled by floating the grids on drops of anti-MT1-MMP (ab-1) and anti-CD9 for 30 min. After washing the grids three times 5 min each with PBS, the bound antibodies were detected with goat anti-rabbit and anti-mouse IgG that were labeled with gold particles, 15 nm (MT1-MMP) and 10 nm (CD9). Finally, the samples were contrasted with uranyl-oxalate (2%, pH 7) for 15 min, washed in distilled water, and embedded in a mixture of 1.8% methyl cellulose and 0.4% uranyl oxalate on ice. After drying, the samples were

examined using transmission electron microscope (Jeol 1200 EX II; Jeol Ltd, Tokyo, Japan).

COLLAGEN DEGRADATION ASSAY

Exosomes from HT-1080 cells were separated in sucrose density gradient, and eight fractions from the top were collected and washed with the degradation assay buffer (0.1 M Tris–HCl containing 0.15 M NaCl and 0.01 M $CaCl_2$). After washing, Brij35 (0.05%) and type I collagen (5 g) from rat tail (Sigma-Aldrich, St. Louis, MO) were added to each fraction in the assay buffer and incubated overnight at 37°C. The digested collagen peptides in the fractions were separated by 10% SDS–PAGE and visualized by Coomassie brilliant blue.

DEGRADATION OF FLUORESCENTLY LABELED GELATIN

Glass coverslips were coated with Oregon green 488-labeled gelatin (Molecular Probes, Leiden, The Netherlands) as instructed by the manufacturer. Exosomes were isolated from MT1-MMP over-expressing HT-1080 cells and immobilized on paramagnetic beads (Dynal Biotech ASA, Oslo, Norway) using a mouse monoclonal antibody against the exosomal marker protein CD9. After 6 h incubation at +4°C the beads were washed twice in PBS and seeded on the gelatin-coated coverslips. GM6001 (10 M) was added into the controls. After 24 h incubation at +37°C the coverslips were fixed with 3% paraformaldehyde and the degradation regions of the gelatin were examined using Zeiss Axioplan 2 epifluorescence microscope (Carl Zeiss Microimaging GmbH, Hamburg, Germany).

ACTIVATION OF MMP-2 BY MT1-MMP CONTAINING EXOSOMES

Gelatin zymography was used to analyze the activation of MMP-2 proenzyme by exosomal MT1-MMP as described [Lohi et al., 1996]. Exosomes from MT1-MMP overexpressing HT-1080 cells were separated in a sucrose density gradient as described above. MMP-2 proenzyme was produced in transiently transfected COS-1 cells, and aliquots of the conditioned cell culture medium were incubated with the exosome containing fractions in the assay buffer (0.1 M Tris–HCl, 0.15 M NaCl, 0.01 M $CaCl_2$, and 0,05% Brij-35, pH 8.0). After 16 h incubation at +37°C, the reactions were terminated with Laemmli sample buffer and the polypeptides separated by electrophoresis in 10% polyacrylamide gels containing gelatin (1 mg/ml). The gels were washed twice with 50 mM Tris/HCl, 5 mM $CaCl_2$, 1 M $ZnCl_2$, and 2.5% Triton X-100 (pH 7.6). After washing once in the buffer without Triton, the gel was incubated overnight at 37°C in the washing buffer containing 1% Triton X-100 and 0.02% NaN_3. The gel was stained with Coomassie brilliant blue R250 and destained with 10% acetic acid in 10% methanol. Zones of proteolytic activity were observed as clear bands in the stained gel.

RESULTS

G361 MELANOMA CELLS RELEASE EXOSOMES THAT CONTAIN MT1-MMP, CD9, TSG101, AND BETA-1 INTEGRIN

Human melanoma (G361) cells were grown in a culture medium containing 5% FCS depleted from exosomes. After 72 h, the cell culture medium was harvested. Cancer cells release from their plasma membranes large, 300–600 nm, vesicles [Hakulinen et al., 2004; Hakulinen and Keski-Oja, 2006]. The cell culture media was ultrafiltered (0.22 m) before high-speed differential centrifugation to exclude the plasma membrane vesicles. The isolated exosomes were further separated from residual membrane fragments and other impurities in a sucrose gradient, where the exosomes flotate to their characteristic density [Thery et al., 2002]. Twelve fractions (1 ml each) were collected from the top of the gradient and assayed by immunoblotting and electron microscopy.

The protein concentrations of exosomal isolates after sucrose density gradient fractionation were usually 0.1–0.2 g for 1–2 × 10^6 G361 cells. Concentrations of 0.3–0.5 g have been described for tumor cells [Wolfers et al., 2001]. The exosomal marker proteins CD9 and TSG101 were detected by immunoblotting of the sucrose gradient fractions to localize in fractions 5–8, which represent sucrose densities from 1.11 to 1.2 g/ml as described previously for exosomes (Fig. 1A) [Raposo et al., 1996; Thery et al., 2002; Valadi et al., 2007]. TSG101 is a protein involved in the endosomal sorting and in the delivery of cellular proteins to multivesicular body, and CD9 belongs to tetraspanins. Both of these proteins are enriched in exosomes [Andre et al., 2002a; Stoorvogel et al., 2002; Thery et al., 2002; Gutwein et al., 2005]. TEM analysis of fractions 3–10 revealed vesicles (<100 nm) with the shape and size characteristic for exosomes in fractions 5–8 (Fig. 1B). The immunoblot of sucrose density gradient fractions for exosomal markers and immunoelectron microscopy results are consistent with each other and indicate that the fractions contain exosomes. Interestingly, by immunoblotting with anti-MT1-MMP antibody, we detected a 60 kDa band of MT1-MMP in the exosome containing fractions 6–7 (Fig. 1A). A 43 kDa band representing the inactive [Lohi et al., 1996], autocatalytically cleaved form of MT1-MMP [Lehti et al., 1998] was also detected in fraction 6. Furthermore, 1-integrin, which in addition to being a major ECM adhesion receptor can interact and co-operate with MT1-MMP [Gálvez et al., 2002], was detected in the exosomal fractions. To define whether the exosomal MT1-MMP is expressed at the surface of exosomes, IEM was carried out. Aliquots of the sucrose gradient fraction 6 from G361 cells were adsorbed on grids and immunolabeled with antibodies to MT1-MMP and CD9. TEM analysis revealed the association of MT1-MMP (15 nm gold) and CD9 (10 nm gold) with flattened, saucer shaped spheres characteristics similar to exosomes (Fig. 1C).

MT1-MMP is cycled between the plasma membrane and cell endocytic compartments. We asked next how MT1-MMP is segregated between cell membranes and exosomes. To get an estimate of the polarization of MT1-MMP between the membranes, we isolated the cell membranes and exosomes from G361 cells and determined their total protein concentrations. Increasing amounts of protein were loaded into the wells of 10% SDS–PAGE, and MT1-MMP, 1-integrin and exosomal marker proteins (TSG101 and CD9) were detected by immunoblotting (Fig. 2). Exosomal MT1-MMP migrated as a 60 kDa band, which was slightly enriched in exosomes when compared to the corresponding band of the cell membrane fraction (Fig. 2A). In addition, a higher molecular weight non-specific band was detected in the cell membrane preparations. 1-integrin was more prominent on the cell membranes than on the exosomes (Fig 2B). As expected, the exosomal marker proteins (CD9 and TSG101) were enriched in exosomes (Fig. 2C,D), and only minor band for CD9 was detected in the cell membrane preparation (Fig. 2D).

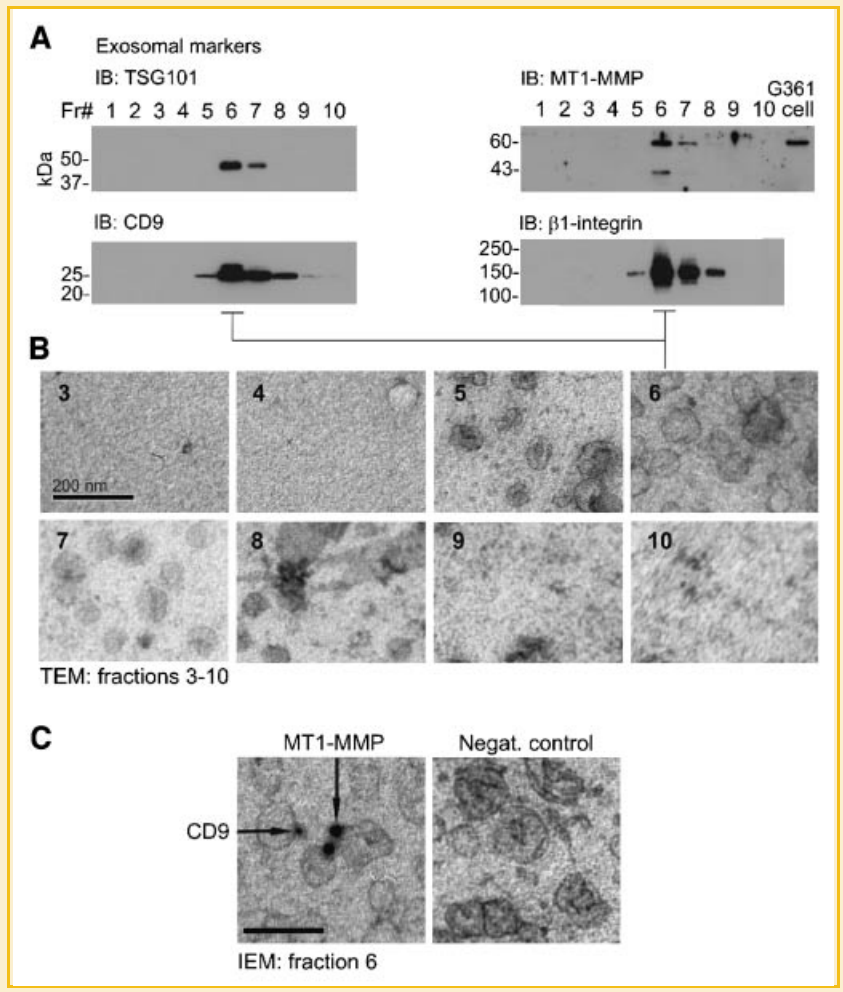

Fig. 1. MT1-MMP and 1-integrin are released to extracellular space on tumor cell exosomes. Exosomes were isolated from G361 cell culture supernatants by ultrafiltration (0.22 m) and differential centrifugation. Ten fractions (1 ml) were collected from the top of the sucrose gradients and washed once in PBS. Polypeptides of the fractions were separated by SDS–PAGE and immunodetected for (A) exosomal marker proteins (CD9 and TSG101) and MT1-MMP. CD9 and TSG101 cosedimented with full length MT1-MMP in fractions 6 and 7. The G361 cell lysate, used as a positive control for MT1-MMP, is shown on the right. B: Aliquots of the gradient fractions (3–10) were adsorbed on gold-coated copper grids and stained with uranyl acetate for transmission electron microscopy analysis (TEM). Exosomes of <100 nm were detected in the fractions 5–8 by TEM colocalizing with fractions immunostained for the exosomal marker proteins. C: Immunoelectron microscopic analysis (IEM) of MT1-MMP and CD9 on exosomes. An aliquot of the exosomes from sucrose gradient fraction 6 isolated from G361 cells was used for the IEM study to analyze the expression of MT1-MMP and the exosomal marker protein CD9. Arrows indicate a gold particle detecting MT1-MMP (15 nm) and CD9 (10 nm). For the negative controls the primary antibodies were replaced with irrelevant antibodies of the same subclass (bar: 100 nm).

EXOSOMAL MT1-MMP IS ENZYMATICALLY ACTIVE

To determine whether the exosomal MT1-MMP was catalytically active, we isolated exosomes from HT-1080 cells stably expressing recombinant MT1-MMP [Lehti et al., 1998]. After sucrose density gradient centrifugation 12 fractions were collected. The MT1-MMP polypeptides of the washed pellets from eight fractions were detected by immunoblotting with anti-MT1-MMP antibodies. A 60 kDa form of MT1-MMP was detected in the immunoblot in the fraction 6 (Fig. 3) like in exosomes isolated from G361 cells. The total cell lysate, which was used as a positive control, contained both the full-length 60 kDa and the proteolytically processed 43 kDa forms of MT1-MMP. To determine whether the exosomes display proteolytic activity for the physiological MT1-MMP substrate we incubated purified type I collagen with aliquots of the washed pellets of the fractions 1–8. The incubation was terminated by Laemmli sample buffer, and the polypeptides were separated by 10% SDS–PAGE. After Coomassie brilliant blue staining, the degradation of type I collagen was observed in the fraction 6 resulting into characteristic peptide fragments (Fig. 3). In the negative control, collagen 1 and 2 chains remained intact and no degradation fragments appeared.

EXOSOMES DEGRADATE OF FLUORESCENTLY LABELED GELATIN

MT1-MMP is concentrated at discrete areas at the cell membranes in migrating cells resulting in enriched MT1-MMP-mediated gelatin degradation at migration sites [Galvez et al., 2001]. To study the degradation of gelatin in situ, we concentrated HT-1080 cell exosomes on paramagnetic beads using an antibody against the

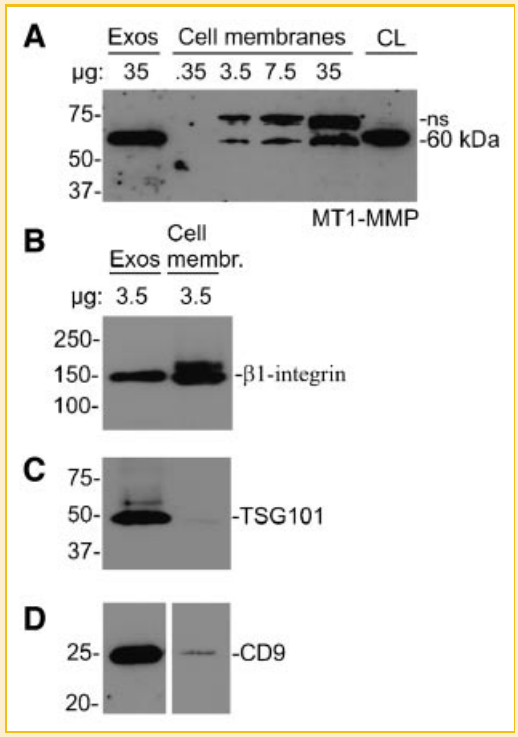

Fig. 2. Segregation of MT1-MMP, CD9, TSG101, and 1-integrin between exosomes and cell membranes. Exosomes and cell membranes were isolated from G361 cells and their total protein concentrations were determined. The indicated amounts of protein (g) were separated by 10% SDS–PAGE and immunodetected for MT1-MMP (A), 1-integrin (B), TSG101 (C), and CD9 (D). Total lysate of G361 cells overexpressing MT1-MMP (CL) was used as a positive control. A 60 kDa band for MT1-MMP was detected in exosomes and the cell membrane preparations (A), a higher molecular weight non-specific band (ns) was detected in the cell membrane samples.

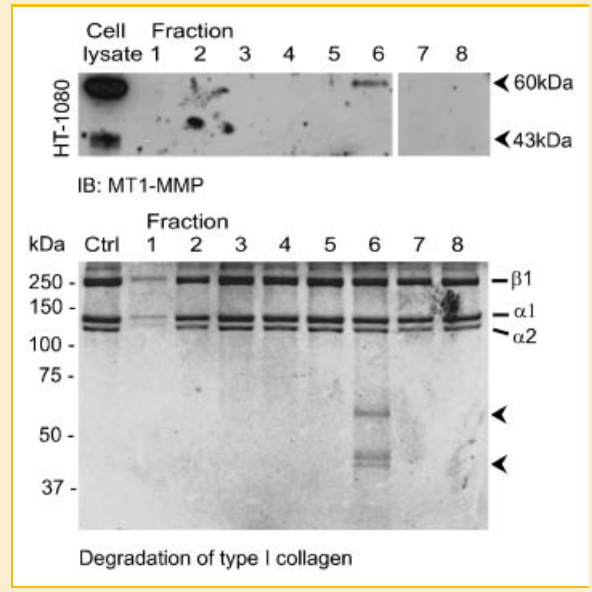

Fig. 3. Exosomes display collagenolytic activity cleaving the physiological MT1-MMP substrate type I collagen. Exosomes were isolated from overexpressing HT-1080 cells stably transfected with MT1-MMP. After sucrose density gradient flotation 8 fractions were collected from the top and washed. Upper panel: Aliquots of the fractions were analyzed by immunoblotting for the expression of MT1-MMP. Total cell lysate was used as a positive control. MT1-MMP (60 kDa) was detected in the fraction 6. Lower panel: Collagen degradation assay. Aliquots of type I collagen were incubated with the fractions 1–8. Exosomes were omitted from the negative control. After incubation overnight at 37°C the digested polypeptides were separated by 10% SDS–PAGE and stained with Coomassie brilliant blue. Type 1 collagen chains are indicated by 1 and 2, and the cleavage products by arrowheads. Activity in fraction 6 digested type I collagen into its characteristic degradation products.

marker protein, CD9. The beads were seeded on coverslips coated with FITC labeled gelatin to visualize the focal degradation of gelatin films by exosomes. Gelatin was degraded around the exosome containing beads and gelatinolysis was observed as dark patches on otherwise homogeneous film (Fig. 4A). As expected, the degradation was inhibited by the metalloproteinase inhibitor GM6001.

MMP-2 IS ACTIVATED BY EXOSOMES

Active MT1-MMP cleaves MMP-2 to an intermediate form resulting to a subsequent autocatalytic cleavage to a fully active MMP-2 [Lehti et al., 2002]. To characterize the activation of MMP-2 by exosomes, we incubated the exosomal fraction 6 from MT1-MMP overexpressing HT-1080 cells with MMP-2 proenzyme expressed in COS-1 cells. Activated form of MMP-2 was detected below the latent form in the zymogram (Fig. 4B). No gelatinolytic bands were detected in the control samples containing exosomes when MMP-2 was omitted. Some autoactivation of MMP-2 could be observed in the MMP-2 control sample incubated with the reaction buffer only.

DISCUSSION

Like a number of proteins MT1-MMP is internalized from the cell surface and cycled to various endocytic compartments through early and recycling endosomes to late endosomes, and recycled to plasma membrane [Remacle et al., 2003]. The small 30–100 nm membrane vesicles, exosomes, originate from late endosomes, that is, multivesicular bodies (MVB) inside the cell, and form by budding inwards from the membrane of MVB into the endosomal lumen. Exosomes are released to the pericellular space upon fusion of the MVB with the cell plasma membrane [Thery et al., 2002]. We therefore, hypothesized that some of the endosomal MT1-MMP could be directed to exosomes for extracellular release.

Exosomes are released by multiple cell types including dendritic cells, B-lymphocytes, platelets, epithelial, and tumor cells [Denzer et al., 2000a; Wolfers et al., 2001; Andre et al., 2002b; Thery et al., 2002]. Exosomes contain multiple but a selected set of proteins including major histocompatibility protein, integrins, tumor antigens, and proteins that probably address exosomes to target cells [Denzer et al., 2000a; Thery et al., 2001, 2002; Wolfers et al., 2001; Andre et al., 2002a]. Furthermore, ovarian carcinoma cells secrete functionally active ADAM-10 and 17 in exosomes [Gutwein et al., 2005; Stoeck et al., 2006]. The mechanisms for the specific sorting of proteins in exosomal membranes are not known. However, the lipid raft-associated proteins are sorted into exosomes [Gassart et al., 2003].

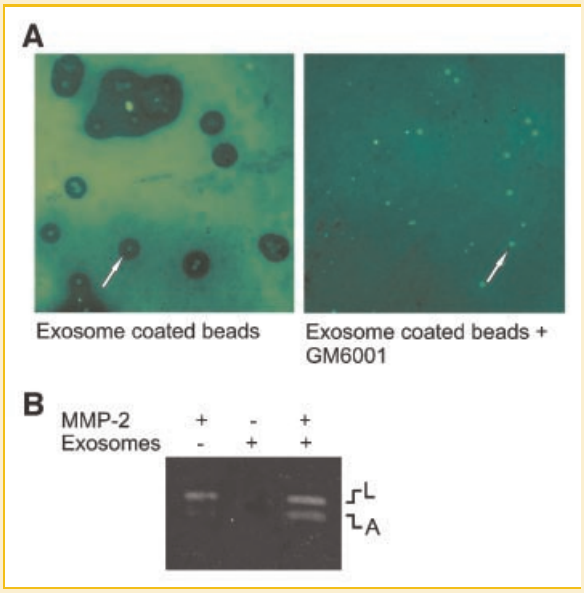

Fig. 4. Exosomes isolated from MT1-MMP overexpressing HT-1080 cells digest fluorescein-labeled gelatin and activate MMP-2 proenzyme. A: Exosomes were captured on paramagnetic beads using an antibody against a protein (CD9) expressed on exosomes. The beads were seeded on coverslips coated with FITC-labeled gelatin. After overnight incubation, the gelatin films were examined using an epifluorescence microscope. Gelatin was degraded around exosome containing beads (indicated by an arrow) and is seen as dark lysis zones. GM6001 (10 M) was used to inhibit MT1-MMP activity in the control. B: Exosomes isolated by sucrose density gradient flotation were incubated with MMP-2 and analyzed by zymography. Latent (L) and activated (A) forms of MMP-2 were detected by zymography after incubation with exosomes. Traces of autoactivated MMP-2 were detected in the untreated control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

We describe here that MT1-MMP is released from HT-1080 fibrosarcoma and G361 melanoma cells into the extracellular space in exosomes as catalytically active full-length enzyme. In addition, we detected the autocatalytically cleaved 43 kDa form (Figs. 1A and 3). Both MT1-MMP and ADAM10 have been observed in large 300–600 nm vesicles originating from the plasma membranes [Taraboletti et al., 2002; Gutwein et al., 2005]. To exclude these we ultrafiltered the cell culture media through 0.22 m pore membrane before high-speed centrifugation and flotated exosomes in a sucrose gradient to their characteristic density to remove the plasma membrane fragments.

We found that MT1-MMP colocalized with the exosomal marker proteins CD9 and TSG101 in the washed gradient fractions by immunoblotting analysis (Fig. 1A). As expected, CD9 and TSG101 were efficiently enriched in exosomes when compared to the cell membranes, where only a minor fraction was detected (Fig. 2C,D). The segregation of the activated 60 kDa form of MT1-MMP was more unbiased between exosomes and cell membranes. Furthermore, only 10% of the protein in exosome preparations was required for the detection of the marker proteins compared to MT1-MMP. A higher molecular weight, non-specific band was detected in the cell membrane preparations, while the band was barely detected in the exosomal fraction using a longer exposure (data not shown). In the longer exposure also the inactive 43 kDa form was detected in the cell membrane, exosome, and MT1-MMP overexpressing cell lysate that was used as a positive control (data not shown).

In accordance with previous findings, 1-integrin was detected in the exosomal fractions [Clayton et al., 2004]. 1-integrin expression was more prominent in the cell membranes than in exosomes (Fig. 2B). Fibroblast, epithelial, and B cell-derived exosomes contain 1-integrin and are able to adhere to type I collagen, fibronectin, and tumor necrosis factor alpha activated fibroblasts, suggesting that exosomes may form an extracellular pool of factors that can bind to extracellular matrix components [Clayton et al., 2004]. MT1-MMP colocalizes with 1- and 3-integrins at cell–cell contacts on endothelial cell membranes grown on collagen I, fibronectin, or fibrinogen [Gálvez et al., 2002]. However, growing of the cells on gelatin and vitronectin resulted in the endocytosis of MT1-MMP [Gálvez et al., 2002]. In adenocarcinoma cells, 1-integrin-mediated adhesion to collagen triggered a polarized trafficking of subcellular MT1-MMP to the plasma membrane in VSV-G/Rab8 positive vesicles [Bravo-Cordero et al., 2007]. The existence of 1-integrin on exosomes may provide a mechanism to transfer focal MT1-MMP activity beyond cell surface by compartmentalizing MT1-MMP and excluding the proteinase inhibitors. MT1-MMP activity could be stored extracellulary in exosomes in the ECM and liberated with various stimuli during physiological tissue remodeling processes providing a novel way to regulate pericellular proteolysis. Indeed, MT1-MMP may undergo intercellular transfer and assemble a multicatalytic complex of stroma-derived MT1-MMP and MMP-2 on the surface of cancer cells [Pei and Weiss, 1996]. MT1-MMP transcripts were exclusively expressed in the stromal cells of the tumors. However, the MT1-MMP protein was observed in cancer cells by immunofluorescence [Sato et al., 1994; Okada et al., 1995]. Recent studies have revealed that exosomes may present a mechanism for the transfer of material from cell to cell [Denzer et al., 2000b; Karlsson et al., 2001; Valadi et al., 2007] by binding or fusing with target cell membranes, which thus acquire new subset of proteins [Thery et al., 2002]. Exosome-mediated membrane transfer between cells has been observed in bone marrow transplantation, where the thymocytes of the donor acquire the MHC molecules of the host [Thery et al., 2002].

Besides its collagen degrading activity MT1-MMP functions as a receptor for pro-gelatinase A (pro-MMP-2) and tissue inhibitor of metalloproteinase-2 (TIMP-2) forming a complex at the cell surface. Activated MT1-MMP cleaves MMP-2 to an intermediate form resulting to a subsequent autocatalytic cleavage to a fully active MMP-2 [Lehti et al., 1998]. The internalized MT1-MMP-bound TIMP-2 is then degraded [Maquoi et al., 2000]. Our attempts to identify TIMP-2 or MMP-2 complexes on exosomes have been unsuccessful (data not shown). However, exosomes isolated from MT1-MMP overexpressing cell line (HT-1080) were able to activate latent MMP-2 in the cell culture supernatants of COS cells (Fig. 4B). Furthermore, exosomes captured on paramagnetic beads were able to digest fluorescein-labeled type I collagen. The capture of exosomes on paramagnetic beads concentrated MT1-MMP activity resulting in effective digestion of gelatin matrix. In addition to its actual function in the degradation of ECM, MT1-MMP in exosomes may participate in the activation of cytokines, as it does on the cell

membranes activating latent TGF-1 [Mu et al., 2002]. Interestingly, tumor-derived exosomes carry membrane associated TGF-1, which can inhibit the cytotoxic functions of natural killer cells contributing to tumor immune escape [Clayton et al., 2007].

Internalization of MT1-MMP from the cell surface and cycling between plasma membrane and cell endocytic compartments regulate the enzyme availability at the cell surface. Earlier MT1-MMP has been shown to be proteolytically released in truncated catalytically active soluble forms (50 and 52 kDa) from the cell membranes [Toth et al., 2002, 2006]. We propose that the release of full length active MT1-MMP on exosomes represents a novel mechanism for cancer cells to target membrane type metalloproteolytic activity into the extracellular space.

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