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Urokinase Plasminogen Activator and Gelatinases Are Associated with Membrane Vesicles Shed by Human HT1080 Fibrosarcoma Cells*

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Membrane vesicles are shed by tumor cells both *in vivo* and *in vitro*. Although their functions are not well understood, it has been proposed that they may play multiple roles in tumor progression. We characterized membrane vesicles from human HT1080 fibrosarcoma cell cultures for the presence of proteinases involved in tumor invasion. By gelatin zymography and Western blotting, these vesicles showed major bands corresponding to the zymogen and active forms of gelatinase B (MMP-9) and gelatinase A (MMP-2) and to the MMP-9·tissue inhibitor of metalloproteinase 1 complex. Both gelatinases appeared to be associated with the vesicle membrane. HT1080 cell vesicles also showed a strong, plasminogen-dependent fibrinolytic activity in ^{125}I fibrin assays; this activity was associated with urokinase plasminogen activator, as shown by casein zymography and Western blotting. Urokinase was bound to its high affinity receptor on the vesicle membrane. Addition of plasminogen resulted in activation of the progelatinases associated with the vesicles, indicating a role of the urokinase-plasmin system in MMP-2 and MMP-9 activation. We propose that vesicles shed by tumor cells may provide a large membrane surface for the activation of membrane-associated proteinases involved in extracellular matrix degradation and tissue invasion.

The shedding of membrane vesicles by tumor cells *in vivo* and *in vitro* has been proposed to be an important feature of malignant transformation (1–3). Membrane vesicles are shed by viable cells *in vitro* (4), and shedding is stimulated by fetal calf serum (5, 6). Although it derives from the plasma membrane, the membrane of shed vesicles appears to have a different lipid composition, which makes it more rigid than the rest of the cell membrane (7). Differences have also been described in the protein composition: alkaline phosphatase and antibody binding activities are more elevated in vesicle membranes than in the cell membrane; D-glutamyl transpeptidase and protein kinase activities, which are high in plasma membranes, are undetectable in shed vesicle membranes (8).

Extracellular vesicles have been proposed as playing a relevant role in the escape of tumors from the immune response (9, 10) and promoting tumor cell invasion (11). Mouse B16 melanoma cell variants with high metastatic potential shed more membrane fragments than poorly metastatic cells (12). Mouse melanoma cells cultured *in vitro* shed a metalloproteinase activity (M_r 59,000) as a component of membrane vesicles (13).

Vesicles from human 8701-BC or MCF-7 breast carcinoma cells carry gelatinolytic activities similar in electrophoretic mobility to progelatinases B (proMMP-9) and A (proMMP-2) (6). These vesicles also inhibit lymphocyte proliferation, their inhibitory effect being neutralized by antibodies to transforming growth factor β (14). The membrane of vesicles from 8701-BC and MCF-7 cells derives from selected areas of the cell membrane. All antigens expressed by the two cell lines are present on the vesicle membrane (15); however, several surface antigens appear to be unevenly distributed, and antibodies to proMMP-9 bind to the vesicles but not to the cell membrane.[1] Thus, shedding of extracellular vesicles may represent an important mechanism of cell-cell and cell-matrix interaction; membrane vesicles may be actively involved in extracellular matrix (ECM)[2] degradation and tissue remodeling.

Tissue remodeling requires the concerted action of a number of extracellular proteinases. Among these enzymes, urokinase plasminogen activator (uPA) and a variety of matrix metalloproteinases (MMPs) play important roles (16–19). Two MMPs, MMP-2 and MMP-9, degrade a variety of ECM components, including type IV collagen, elastin, and proteoglycans as well as denatured collagens (*i.e.* gelatins). MMP-2 also digests type I collagen, in addition to laminin and fibronectin (18, 20, 21). Both uPA and MMPs are secreted as inactive zymogens (pro-uPA, proMMPs) and activated extracellularly by limited proteolysis (22). Plasmin degrades a variety of ECM components and activates several MMPs with different substrate specificities, including MMP-1 (interstitial collagenase), MMP-3 (stromelysin 1), and to a certain extent, MMP-9 (20, 23–25). The physiological mechanism(s) of proMMP-2 and proMMP-9

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[2] The abbreviations used are: ECM: extracellular matrix; MMP: matrix metalloproteinase; FCS: fetal calf serum; MT-MMP: membrane-type metalloproteinase; PA: plasminogen activator; PBS: phosphate buffer saline; PI-PLC: phosphatidylinositol phospholipase C; TIMP: tissue inhibitor of metalloproteinase; uPA: urokinase plasminogen activator; uPAR: urokinase receptor.

activation are not fully understood.

uPA, plasmin(ogen), and some MMP activities are localized and/or modulated on the cell surface (26–32). uPA binds to a high affinity cell membrane binding site (uPAR) through a specific N-terminal sequence of its noncatalytic chain (29) and remains active on the cell surface for several hours (30–32). Binding of uPA to uPAR accelerates plasminogen activation on the cell surface and localizes the enzyme to focal contact sites (33–35). Low affinity, high capacity binding sites for plasminogen or plasmin are present on the surface of most cells (36–38). MMP-2 interacts with the cell membrane at specific regions (podosomes or *invadopodia*) involved in cell invasion (39, 40). Recently, MMP-2 has been shown to bind to the avβ3 integrin (41). Cell membrane components are involved in both the binding and activation of proMMP-2 (26–28, 42–45). The transmembrane proteins MT-MMPs (membrane-type MMPs) activate MMP-2 under certain conditions but have no effect on MMP-9 (43–45). Different M_r forms of MMP-9 are also associated with the cell surface (46). Cell-bound MMP-2 and MMP-9 are activated by plasmin; in contrast, in the absence of cells, plasmin degrades both gelatinases (46).

Here we report that vesicles from human HT1080 fibrosarcoma cells have membrane-bound gelatinases and uPA. Addition of plasminogen to vesicles results in gelatinase activation, indicating that plasmin may represent a mechanism for modulating gelatinase activity on the vesicle surface. Our results suggest that vesicles shed by tumor cells may provide a large membrane surface for the activation of membrane-bound proteinases involved in tissue remodeling.

EXPERIMENTAL PROCEDURES

Materials—Pure human proMMP-2, proMMP-9, TIMP-1, polyclonal antibodies to these proteins, and a human recombinant proMMP-3 (ΔC) that lacks the C-terminal domain but has the same activity as the wild type have been described (47–49). The monoclonal antibody IgG1 to the kringle domain of human uPA was kindly provided by Lepetit (Gerenzano, Italy). Bovine plasminogen and human fibrinogen were purified as described (50). Pure human uPA (Ukidan; 100,000 IU/mg) was purchased from Serono (Rome, Italy); gelatin-Sepharose from Pharmacia Biotech AB (Uppsala, Sweden); phosphatidylinositol phospholipase C (PI-PLC) from Boehringer Mannheim; gelatin, agarose, Tween 20, *p*-aminophenylmercuric acetate, and amiloride from Sigma; biosynthetically [^{3}H]proline-labeled type IV collagen from Amersham Life Science, Inc. Human fibrinogen was labeled with ^{125}I (Amersham) using Iodogen (Pierce) as described (51).

Cell Cultures—Two independent strains of human HT1080 fibrosarcoma cells were used. One strain, which we denominated HT1080/PA, was recently obtained from Istituto Zooprofilattico (Brescia, Italy) and grown in tissue culture flasks (Falcon, Becton Dickinson) in RPMI 1640 medium (Mascia Brunelli, (Milan, Italy)) supplemented with 10% fetal calf serum (FCS, Celbio Hyclone, (Milan, Italy)). The other strain, denominated HT1080/PV, was originally obtained from the American Type Culture Collection and grown in our laboratories for several years in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% FCS and 2 mM L-glutamine. The cells were negative for mycoplasma contamination as routinely tested by Hoechst 33258 (Sigma) staining.

Isolation of Membrane Vesicles from Cell-conditioned Medium—Vesicles were prepared from cell-conditioned medium as described (6). Briefly, after 24 h of incubation with subconfluent cells in the presence or absence of FCS, conditioned medium was centrifuged at 500 $\times$ *g* for 10 min and at 800 $\times$ *g* for 15 min. The supernatant was centrifuged at 100,000 $\times$ *g* for 1 h at 4 °C, and the pelleted vesicles were resuspended in phosphate-buffered saline (PBS). Protein concentration was measured by the Bradford micro assay method (Bio-Rad) using bovine serum albumin (Sigma) as a standard.

Electron Microscopy—Aliquots of vesicles were applied on colloidal-coated grids, negatively stained with 1% phosphotungstic acid, pH 7.2, and observed with a Philips EM 420 electron microscope.

Preparation of Cell Extracts and Conditioned Media—Confluent cell cultures were washed twice with PBS to remove residual FCS and incubated for 16–24 h in serum-free medium with or without the indicated concentrations of plasminogen. The culture supernatant was harvested, and cellular debris was removed by centrifugation at 500 $\times$ *g* for 10 min at 22 °C. The cells were washed twice with PBS, lysed for 10 min on ice with 0.5% Triton X-100 in 0.1 M Tris-HCl, pH 8.1, under constant shaking, and then scraped with a rubber policeman. Following centrifugation at 800 $\times$ *g* for 10 min at 4 °C, protein concentration in the cell lysates was measured by the BCA protein assay reagent using BSA as a standard. Conditioned media were concentrated in Centricon C-10 (Amicon, Inc.) for 1 h at 4 °C. Conditioned media and cell extracts were immediately processed for proteinase assays.

Gelatin-Sepharose Chromatography and Gelatin Zymography—Purified vesicles (10–40 μg of protein) were resuspended in nonreducing Laëmmli buffer and loaded on SDS-3–7.5% polyacrylamide gels containing 1 mg/ml gelatin. Where indicated, vesicles (~10–20 μg of protein in 500 μl of PBS), cell extracts (1 ml), or conditioned media (4 ml) were concentrated with gelatin-Sepharose before gelatin zymography. For this purpose, the samples were incubated at 4 °C for 1 h in an end-over-end mixer with 20–50 μl of gelatin-Sepharose equilibrated with 50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.02% (v/v) Tween 20, 10 mM EDTA, pH 7.6 (52). After four washes with 1 ml of equilibration buffer containing 200 mM NaCl, the beads were resuspended in 30 μl of 4 $\times$ nonreducing Laëmmli buffer and loaded on SDS-3–7.5% polyacrylamide gels containing 1 mg/ml gelatin. After electrophoresis, the gels were washed twice with 200 ml of 2.5% (v/v) Triton X-100 for 1 h at 22 °C to remove SDS and three times with H_2O for 5 min to remove Triton X-100. The gels were incubated at 37 °C for 24–48 h in 50 mM Tris-HCl, 200 mM NaCl, 20 mM $CaCl_2$, pH 7.4, with or without the addition of 10 mM EDTA, 1 mM 1,10-phenanthroline, 1 mM phenylmethylsulfonyl fluoride, or 1 mM *N*-ethylmaleimide, stained overnight with Coomassie Brilliant Blue R-250 0.5% (w/v) in 45% (v/v) methanol, 10% (v/v) acetic acid, and destained in the same solution without dye (53). The M_rs of the lysis bands were determined by reference to high molecular mass standards (29–205 kDa, Sigma).

Casein Zymography for Plasminogen Activators—Zymography for uPA was performed as described (53, 54) using agar overlay gels containing 3% nonfat dry milk and 40 μg/ml pure bovine plasminogen with or without addition of 100 μg/ml aprotinin.

Western Blotting—Twenty μg of vesicle protein was electrophoresed in a SDS-3–7.5% polyacrylamide gel under nonreducing conditions and transferred onto a nitrocellulose membrane (Hybond, Amersham) as described (55). The membrane was saturated with 5% horse serum, 0.1% Tween 20 in PBS for 2 h, incubated with the indicated antibody (1:200) for 1 h, and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:7500, Sigma) for 1 h at room temperature. Immunocomplexes were visualized by the ECL Western blotting kit (Amersham) using Hyperfilms.

Assay for Type IV Collagen Degradation—Eight μg of vesicle protein in 1 ml of medium were added to ^{3}H type IV collagen-coated microculture wells (2 cm^2; 3,000 dpm/well) (56). After 16 h of incubation at 37 °C, 800-μl aliquots of the supernatants were collected, and radioactive degradation products were measured in a Camberra Packard liquid scintillation counter. Samples and controls were assayed in duplicate.

Assays for Plasminogen Activator (PA) Activity—Five μg of vesicle protein, 1–10 μg of cell extract protein, and 10–50 μl of cell-conditioned medium were tested for PA activity by the ^{125}I-fibrin assay (57) in the presence of 4 μg/ml pure plasminogen. Amiloride (1 mM) was added to control wells to specifically inhibit uPA activity. Where indicated, uPA activity was measured by reference to a standard curve obtained with pure human uPA (Ukidan; 100,000 IU/mg).

PI-PLC Treatment—To remove uPA·PAR complexes from the membrane, vesicles were incubated with 900 milliunits of PI-PLC for 1 h at 37 °C in 1 ml of Dulbecco's modified Eagle's medium with Hepes, pH 7.4. As a control, vesicles were incubated with medium alone. At the end of incubation, vesicles were centrifuged at 3,000 $\times$ *g* for 1 h in Centricon C-500 tubes (cutoff, 500 kDa), and the medium recovered in the lower chamber was concentrated to 40 μl in Centricon C-10 tubes (Amicon). The vesicles retained in the upper chamber of the Centrispeed tubes, the concentrated PI-PLC or control medium washings, were tested for PA activity as described above.

Triton X-114 Extraction—Vesicles (200 μg) were resuspended in 1 ml of 1.5% Triton X-114 in 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$ and incubated for 20 min at 4 °C and then 5 min at 37 °C. After centrifugation at 5,000 $\times$ *g* for 5 min at room temperature, the detergent phase was separated from the aqueous phase. The phase separation was repeated three times. Both the detergent and the aqueous phase were immediately processed for gelatin-Sepharose chromatography as described above.

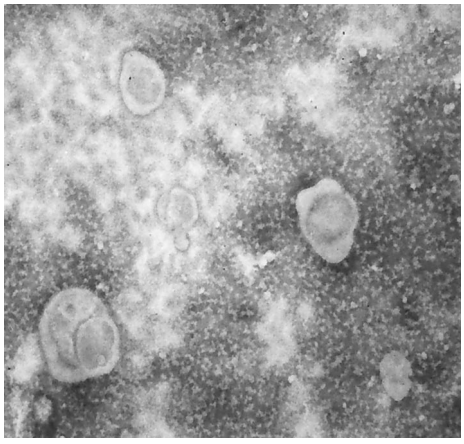

FIG. 1. **Structure of isolated vesicles.** Vesicles were purified from HT1080 vesicles as described under "Experimental Procedures" and observed with a Philips EM 420 electron microscope after negative staining with 1% phosphotungstic acid. The diameter of observed vesicles ranges from 100 to 400 nm. Magnification, × 45,000.

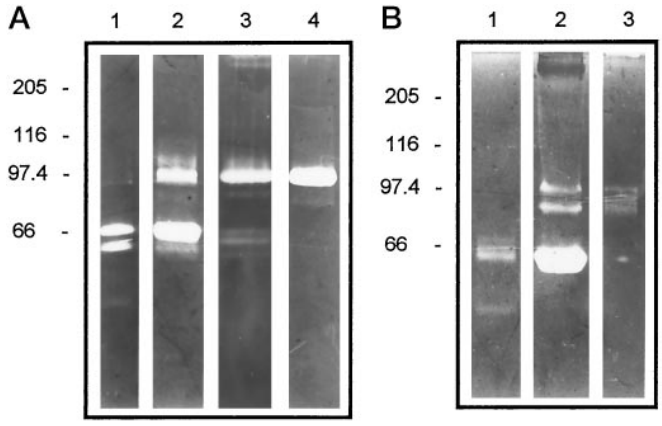

FIG. 2. **Gelatin zymography of vesicle-associated gelatinases.** Vesicles were purified from cell-conditioned medium containing 10% FCS and analyzed by gelatin zymography as described under "Experimental Procedures." *A*, *lane 1*, purified proMMP-2 and MMP-2 (0.5 ng); *lane 2*, vesicles from HT1080/PA cells (20 μg); *lane 3*, vesicles from HT1080/PV cells (20 μg); *lane 4*, purified proMMP-9 (1 ng). *B*, samples preincubated with 1 mM *p*-aminophenylmercuric acetate for 3 h at 37 °C. *Lane 1*, pure proMMP-2 (5 ng); *lane 2*, HT1080/PA vesicles (40 μg); *lane 3*, pure proMMP-9 (4.5 ng). Molecular masses are shown in kDa on the *left* of each *panel*.

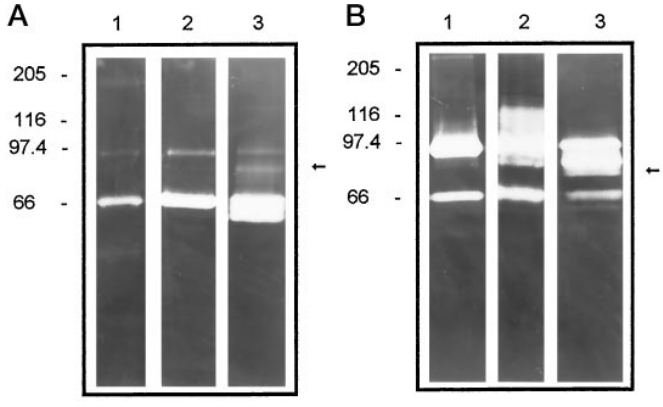

FIG. 3. **Zymographic analysis of gelatinases associated with vesicles, cell extracts, and conditioned media of HT1080/PA and HT1080/PV cells.** Vesicles were purified and analyzed by gelatin zymography as described under "Experimental Procedures." Conditioned media and cell extracts were concentrated with gelatin-Sepharose before gelatin zymography, as described under "Experimental Procedures." *A*, HT1080/PA cells; *B*, HT1080/PV cells. *Lane 1*, vesicles (10 and 20 μg); *lane 2*, conditioned medium (200 and 400 μl); *lane 3*, cell extracts (400 and 470 μg). Molecular masses are shown in kDa on the *left* of each *panel*. *Arrows* indicate the 79-kDa form of MMP-9.

RESULTS

Morphology of Vesicles and Effect of Serum on Shedding—Vesicles were isolated from medium conditioned by HT1080/PA or HT1080/PV cells grown in the presence or absence of 10% FCS. The morphology of the vesicles is shown in Fig. 1. As was previously observed with mammary carcinoma cell-derived vesicles (6), their shape is roughly spherical, and their dimensions are variable. Vesicles recovered from serum-free or from complete medium conditioned by the two HT1080 cell strains had comparable morphology. HT1080/PA and HT1080/PV cells also shed similar amounts of vesicles: 3×10^7 cells shed ~150 μg of vesicle protein/24 h in the presence of 10% FCS and only 30 μg in serum-free medium.

MMP-2, MMP-9, and MMP-9·TIMP-1 Complex Are Associated with Vesicles—By gelatin zymography (Fig. 2*A*), vesicles from both HT-1080/PA and HT1080/PV cells grown in the presence of 10% FCS showed two major bands that co-migrated with purified proMMP-9 and proMMP-2 and minor bands of about 82 kDa and 62 kDa. The latter bands co-migrated with those obtained by treatment of the vesicles with *p*-aminophenylmercuric acetate, which activates the gelatinases *in vitro* (Fig. 2*B*). The development of all the bands was inhibited by EDTA or 1,10-phenanthroline but not by phenylmethylsulfonyl fluoride or *N*-ethylmaleimide (data not shown).

The bands corresponding to active MMP-2 and MMP-9 associated with vesicles shed in the presence of serum were absent in vesicles from serum-free cultures. Vesicles and conditioned media from both HT1080 cell strains showed comparable bands. In contrast, cell extracts (Fig. 3) showed an additional band of ~79 kDa, which may represent nonglycosylated MMP-9 (24). This observation is in agreement with previous findings that this form of MMP-9 is exclusively intracellular (46). High levels of MMP-2 and very low levels of MMP-9 were associated with cell extract, conditioned medium, or vesicles from HT1080/PA cells; in contrast, HT1080/PV cell extracts, conditioned medium, and vesicles showed levels of MMP-9 considerably higher than those of MMP-2.

Fig. 4*A* shows Western blotting analysis of vesicles with anti-MMP-9 and anti-TIMP-1 antibodies. In addition to recognizing the 92-kDa proenzyme, anti-MMP-9 antibody reacted strongly with a large band of ~140 kDa. This band was also stained by anti-TIMP-1 antibody, indicating that it represents proMMP-9·TIMP-1 complex. Anti-TIMP-2 antibody did not recognize any vesicle protein (data not shown). To confirm the presence of the proMMP-9·TIMP-1 complex, vesicles were treated with recombinant MMP-3, which activates proMMP-9 (25, 58). As shown in Fig. 4*A*, treatment with MMP-3 resulted in decreased size and intensity of the 140-kDa band and in the generation of a band that co-migrated with purified TIMP-1 and was recognized by anti-TIMP-1 antibody. By zymography, vesicles incubated with MMP-3 had more intense 82-kDa and 62-kDa bands (data not shown). A 140-kDa band could also be detected by zymography of vesicles after repeated freezing and thawing (Fig. 4*B*).

MMP-9 and MMP-2 Are Bound to the Vesicle Membrane—Vesicle-associated gelatinases could be inside the vesicles or represent integral membrane proteins or surface-bound enzymes. To discriminate between these hypotheses, we used two different approaches. In one set of experiments, we attempted to purify the gelatinases by gelatin-Sepharose chromatography of intact vesicles as described under "Experimental Procedures." As shown in Fig. 5*A*, MMP-9 and MMP-2 were entirely recovered in the gelatin-Sepharose-bound fraction. By silver

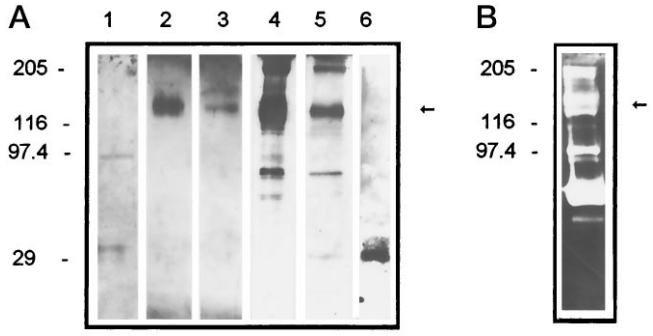

FIG. 4. **Gelatinase·TIMP-1 complex associated with vesicles.** *A*, Western blotting analysis of vesicle proteins with anti-proMMP-9 (*lanes 1–3*) and anti-TIMP-1 (*lanes 4–6*) polyclonal antibodies. *Lane 1*, pure proMMP-9 (10 ng); *lanes 2* and *4*, HT-1080/PA vesicles (20 μg); *lanes 3* and *5*, HT1080/PA vesicles (20 μg), preincubated with 9 ng of human recombinant MMP-3 (ΔC) for 3 h at 37 °C; *lane 6*, pure TIMP-1 (50 ng). *B*, gelatin zymography after freezing and thawing of HT1080 vesicles (20 μg). Western blotting and zymography were carried out as described under "Experimental Procedures." *Arrows*, MMP-9·TIMP-1 complex.

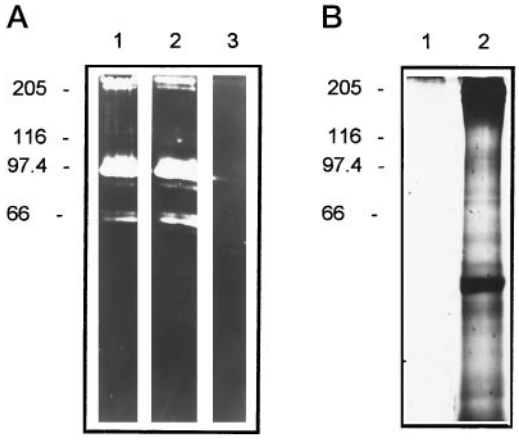

FIG. 5. **Gelatin-Sepharose chromatography of vesicles.** Vesicles (20 μg) were incubated with 20 ml of gelatin-Sepharose in an end-over-end mixer for 1 h at 4 °C as described under "Experimental Procedures." The beads were resuspended in nonreducing Laëmmli buffer, and the supernatant was concentrated in a Centricon C-10 tube. The samples were run in a SDS-7.5% polyacrylamide gel containing 1 mg/ml gelatin or in a SDS-7.5% polyacrylamide gel. The former gel was processed for zymography; the latter was stained by the silver nitrate method as described under "Experimental Procedures." A comparable amount of untreated vesicles was used as a control. *A*, Gelatin zymography of untreated vesicles (*lane 1*), gelatin-Sepharose-bound proteins (*lane 2*), and unbound proteins (*lane 3*). *B*, silver nitrate staining of gelatin-Sepharose-bound proteins (*lane 1*) and unbound proteins (*lane 2*).

nitrate staining, this fraction contained no detectable protein; all vesicle protein being associated with the unbound fraction (Fig. 5*B*). Incubation of vesicles with gelatin-Sepharose under these conditions did not result in significant lysis, as assessed by electron microscopy. In addition, the presence of 10 mM EDTA in the gelatin-Sepharose buffer prevented vesicle binding to gelatin through integrins or other adhesion molecules possibly associated with the vesicle membrane. Therefore, this result indicated that gelatinases are bound to the vesicle surface and accessible to the substrate. In a second set of experiments, we extracted vesicles with Triton X-114, which affords separation of detergent-soluble, integral membrane proteins (59). As shown in Fig. 6, most of the gelatinases were recovered in the aqueous phase; however, significant amounts of proMMP-9 and proMMP-2 were also recovered in the detergent phase. This result indicated that the gelatinases are associated with the vesicle membrane, possibly through relatively weak binding to integral membrane proteins.

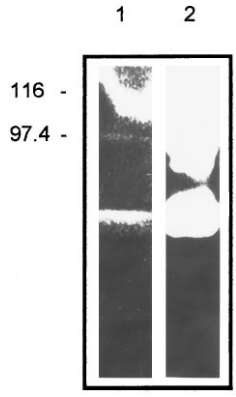

FIG. 6. **Triton X-114 extraction of vesicle gelatinases.** Vesicles (200 μg) were treated with Triton X-114 as described under "Experimental Procedures." The detergent (*lane 1*) and the aqueous (*lane 2*) phases were concentrated with gelatin-Sepharose and analyzed by gelatin zymography as described under "Experimental Procedures."

uPA·uPAR Complexes Are Present on the Vesicle Membrane—To test if PA activity is also associated with vesicles, cell extracts and vesicles from HT1080/PA and HT108/PV cells grown in the presence or in the absence of FCS were tested by the ^{125}I-fibrin assay. The vesicles showed a relatively high PA activity that was completely inhibited by 1 mM amiloride, a specific inhibitor of uPA (60) (Fig. 7*A*). Casein zymography in the presence of plasminogen showed a band of 55 kDa whose activity was inhibited by aprotinin, an inhibitor of serine proteinases. Western blotting of vesicle protein also showed a 55-kDa band that co-migrated with pure human uPA (Fig. 7*B*). Thus, the PA activity of vesicles is exclusively associated with uPA. The specific uPA activity (milliunits/μg of protein) of vesicles from serum-free cultures was twice as high as that of cell extracts (Fig. 8). However, the specific activity of vesicles shed in the presence of FCS was greatly reduced, suggesting complex formation between vesicle uPA and serum inhibitor(s).

To test whether uPA is contained inside the vesicles or bound to their surface, vesicles were treated with phosphatidylinositol-specific phospholipase C, which releases uPAR from the cell membrane (61). The vesicles were then separated from the medium by dialysis in MST-2 Centrispeed concentrators as described under "Experimental Procedures." As shown in Fig. 9, the PA activity of vesicles incubated with control medium without PI-PLC was retained by the dialysis membrane, showing that it was associated with the vesicles. In contrast, the PA activity of vesicles treated with PI-PLC was completely recovered in the dialysate and thus in soluble form. Electron microscopy showed that the PI-PLC treatment did not result in lysis or significant morphological changes in the vesicles (data not shown). Therefore, uPA is bound to the vesicle membrane through its high affinity interaction with uPAR. The enzyme may be bound on the external or on the internal surface of the vesicle membrane: in either case, uPA is accessible to the substrate, as shown by the ability of the vesicles to activate exogenously added plasminogen.

Vesicle-associated Progelatinases Are Activated by Plasmin—Gelatin zymography of vesicles from serum-free medium showed that the gelatinases were predominantly in proenzyme form. In contrast, vesicles from cells grown in the presence of 10% FCS showed the presence of active forms, suggesting that the progelatinases could be activated by serum components. To test the hypothesis that plasmin could be involved in the activation of vesicle-associated gelatinases, we measured the ability of vesicles isolated from serum-free medium to degrade ^{3}H type IV collagen films in the presence or absence of plasminogen. As shown in Fig. 10*A*, low type IV collagen degradation

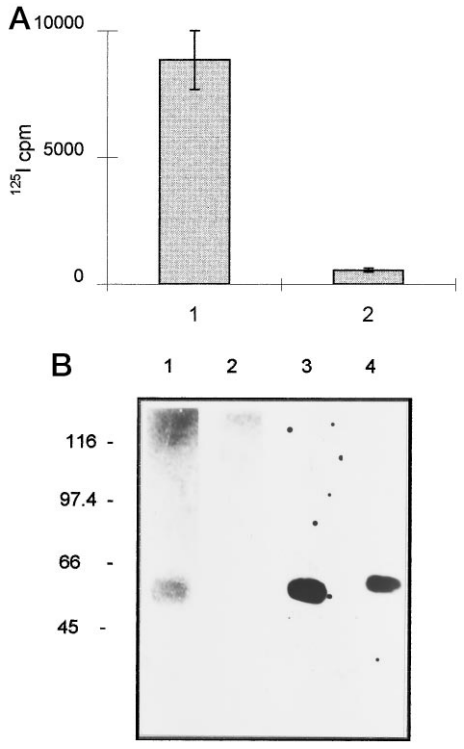

FIG. 7. **Vesicle-associated PA activity.** *A*, HT1080/PV vesicles (5 μg of protein) were tested for PA activity by the ^{125}I-fibrin assay described under "Experimental Procedures" in the absence or in the presence of 1.0 mM amiloride. The values on the *ordinate* are ^{125}I cpm after subtraction of the radioactivity released by control buffer with plasminogen alone. *1*, without amiloride; *2*, with 1.0 mM amiloride. Mean and experimental variability from a representative experiment are shown. *B*, characterization of vesicle-associated PA activity. HT1080/PA vesicles (20 μg) were characterized by casein zymography (*lanes 1* and *2*) and by Western blotting with anti-uPA antibody (*lanes 3* and *4*) as described under "Experimental Procedures." *Lane 1*, zymography without aprotinin; *lane 2,* zymography with 100 μg/ml aprotinin; *lane 3*, vesicles; *lane 4*, pure uPA (50 ng)

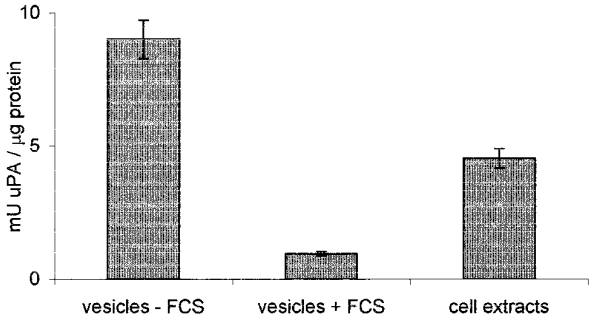

FIG. 8. **Quantitative comparison of the uPA activity associated with HT1080/PV vesicles and cell extracts.** Vesicles (5 μg) and cell extracts (5 μg) were tested for PA activity by the ^{125}I-fibrin assay. The values on the *ordinate* are IU, as determined by reference to a standard curve obtained with pure uPA as described under "Experimental Procedures." Mean and experimental variability from a representative experiment are shown.

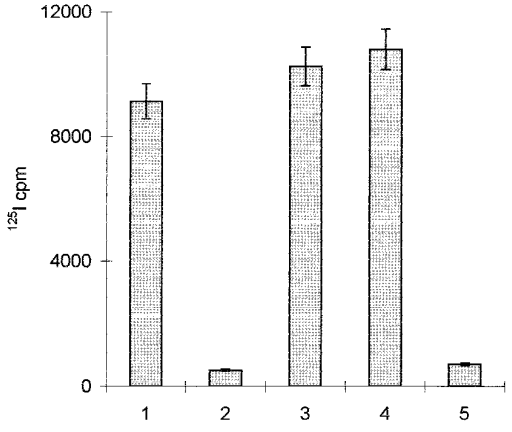

FIG. 9. **Effect of PI-PLC on vesicle-associated PA activity.** HT1080/PV vesicles (5 μg of protein) were treated with PI-PLC (900 milliunits/ml) or with control medium for 1 h at 37 °C. After centrifugation in MST-2 Centrispeed concentrators with 200-kDa cutoff to separate the vesicles from the medium, the material retained by the dialysis membrane and the dialysate were assayed for PA activity by the ^{125}I-fibrin assay as described under "Experimental Procedures." The values on the *ordinate* are ^{125}I-fibrin cpm after subtraction of the radioactivity released by control buffer without vesicles. *1*, untreated vesicles; *2* (retentate) and *3* (dialysate), vesicles treated with PI-PLC; *4* (retentate) and *5* (dialysate), vesicles treated with control medium. Mean and experimental variability from a representative experiment are shown.

was measured in the absence of plasminogen. Addition of 4 μg/ml pure plasminogen increased ^{3}H type IV collagen degradation about 3-fold. In the absence of vesicles, an equivalent concentration of plasmin had no collagenolytic activity.

To confirm the hypothesis that plasmin activates vesicle-associated gelatinases, vesicles recovered from cells grown in serum-free medium in the absence or presence of pure plasminogen (4 μg/ml) were analyzed by gelatin zymography. As shown in Fig. 10*B*, the gelatinases associated with vesicles shed in the absence of plasminogen were only in proenzyme form. In contrast, in the presence of plasminogen, the vesicles possessed both the pro- and the active forms of the gelatinases. In other experiments, vesicles shed in serum-free medium were incubated with pure plasminogen at 37 °C for 3 h. This treatment resulted in the generation of active gelatinase bands (Fig. 10*C*), indicating that plasmin mediates the activation of vesicle-associated MMP-9 and MMP-2.

DISCUSSION

The data reported show that membrane vesicles shed by HT1080 human fibrosarcoma cells have gelatinases and uPA associated to their surface. This conclusion is based on gelatin and casein zymography analyses, activity assays, and Western blotting with specific antibodies. The presence of gelatinolytic enzymes in vesicles shed by tumor cells appears to be a general phenomenon. We have previously detected gelatinases in vesicles shed by two breast carcinoma cell lines (6) and in vesicles shed by ovarian carcinoma cells both *in vivo* and *in vitro*.[3]

These results add further evidence for a role of membrane vesicles in tissue remodeling. Although all vesicles we have analyzed by gelatin zymography show similar patterns of gelatinase activities, we found large differences in the amount of vesicles shed by different cell lines and in the relative amount of proteolytic enzymes they carry. The behavior of cells grown in serum-free medium also varies among different cell lines: some cell lines shed almost undetectable amounts of vesicles, whereas others show a less pronounced serum dependence of vesicle shedding. Moreover, whereas vesicles shed in serum-free medium by some ovarian carcinoma cell lines are rich in gelatinolytic enzymes, vesicles shed under the same conditions by two breast carcinoma cell lines are almost devoid of gelatinolytic activity (6).

Our two strains of HT1080 cells shed relatively large amounts of gelatinase-rich vesicles both in the presence and absence of serum. However, whereas vesicles recovered from

[3] V. Dolo, S. Canevari, A. Consiglio, A. Ginestra, P. Pizzurro, F. M. Romano, and M. L. Vittorelli, unpublished results.

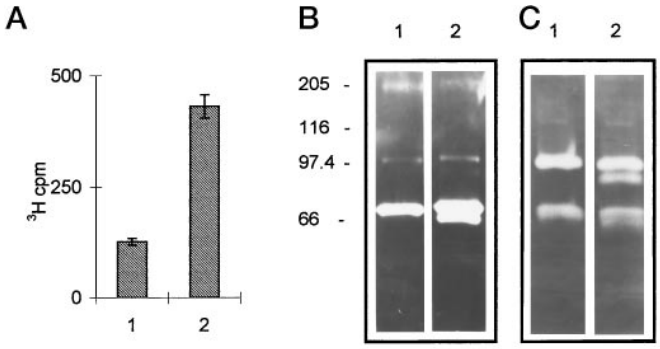

FIG. 10. **Effect of plasminogen on vesicle-associated gelatinases.** Vesicles (8 μg) obtained from HT1080/PA cells grown in serum-free medium in the absence or in the presence of 4 μg/ml plasminogen were tested by the ^{3}H type IV collagen degradation assay (*A*) or by gelatin zymography (*B*) as described under "Experimental Procedures." *A* and *B*, *1*, vesicles from cells grown in the absence of plasminogen; *2*, vesicles from cells grown in the presence of plasminogen. The values shown in *A* are ^{3}H type IV collagen cpm after subtraction of the radioactivity released by control buffer without vesicles. *C*, vesicles obtained from serum-free HT1080/PV cell cultures were incubated in PBS with or without addition of 4 μg/ml plasminogen for 3 h at 37 °C. *Lane 1*, no plasminogen; *lane 2,* with plasminogen.

complete medium showed lytic bands corresponding to both the proenzyme and active forms of the gelatinases, vesicles recovered from serum-free medium showed gelatinases that were exclusively in pro- forms. This finding indicated that serum components are required for progelatinase activation.

The catalytic activity of gelatinases is tightly regulated at three levels: gene transcription, proenzyme activation, and inhibition by specific tissue inhibitors (TIMPs). Western blotting of vesicles from HT1080 cells showed large amounts of a high M_r TIMP-1·proMMP-9 complex. This complex was not detected by gelatin zymography of fresh vesicles but could be evidenced after repeated freezing and thawing. In addition, the complex could partially be disrupted by the addition of MMP-3, which activates proMMP-9 (25, 58).

Our experiments also show that the gelatinases are associated with the vesicle membrane. This conclusion is based on the ability of gelatin-Sepharose to bind vesicle-associated gelatinases under conditions in which vesicle integrity was maintained and integrin-ligand binding was prevented by EDTA. The great majority of vesicle protein remained in the unbound fraction, indicating that gelatinase interaction with the vesicle surface is reversible. When vesicle proteins were extracted with Triton X-114, the gelatinases were mostly recovered in the aqueous phase. However, small amounts of gelatinases were also recovered in the detergent phase. Thus, vesicles carry large amounts of gelatinases that are predominantly associated with the vesicle surface as peripheral membrane proteins and are likely bound to integral membrane components from which they dissociate slowly. MMP-2 has recently been reported to bind to avβ3 integrin (41). A similar interaction may be responsible for gelatinase binding to the vesicle. This hypothesis and the characterization of the vesicle binding site for MMP-9 certainly deserves further investigation.

Vesicles appear to lack the 79-kDa form of MMP-9 associated with cell extracts. The M_r of this polypeptide is consistent with that of nonglycosylated proMMP-9, as predicted from its amino acid sequence (24). This form of MMP-9 has recently been shown to be intracytoplasmic, whereas the 92- and 84-kDa forms of this enzyme appear to be located to the surface of HT1080 cells (46). Because the levels of gelatinases associated with vesicles shed in the presence of serum are consistently higher than those of vesicles recovered from serum-free medium, it is conceivable that not only cell-derived but also serum gelatinases can associate with binding sites on the vesicle membrane. Thus, these findings suggest that the gelatinases are first secreted and then part of the secreted molecules associate with binding sites on the vesicle membrane.

^{125}I-Fibrin assays, casein zymography, and Western blotting with specific antibodies showed that HT1080 cell-derived vesicles carry high amounts of uPAR-bound uPA. The fibrinolytic activity of HT1080 vesicles shed in complete medium was lower than that of vesicles shed in serum-free medium and increased after acid washing (data not shown). This finding suggests that vesicles may bind a serum inhibitor(s) of uPA that is partially released by acid treatment. Other experiments are needed to clarify the molecular nature of this negative regulator(s) of the proteolytic cascade.

The presence of uPA·uPAR complexes on the vesicle membrane is of particular importance. The role of uPAR-bound uPA in plasmin generation and ECM degradation has been demonstrated in several physiological and pathological settings (19). The concomitant binding of pro-uPA to uPAR and of plasminogen to binding sites on the plasma membrane strongly enhances plasminogen activation (62). Plasmin catalyzes the conversion of pro-uPA into active uPA, leading to a positive feedback regulation of the overall reaction (63); in addition, it can activate several proMMPs (19, 25).

Our data show that the gelatinases and uPA are associated with the vesicle membrane. However, our finding that the vesicles remain intact and sealed under the experimental conditions we used does not elucidate whether these enzymes are bound to the external or to the internal surface of the membrane. It is possible that the vesicles open and release their content, then reseal immediately; or *in vitro* conditions render them permeable to substrates. Whereas we cannot rule out these hypotheses, our experiment with PI-PLC shows that vesicles incubated with control medium have uPA activity exclusively associated with the nondialyzable fraction (Fig. 9). If the vesicle membrane were permeable and uPA present in the internal compartment in soluble phase, the enzyme should equally partition inside the vesicle and in the culture medium. Thus, our results strongly indicate that uPA and the gelatinases are associated with the external surface of the vesicles. Whichever their localization, these vesicle-associated proteinases are accessible to external substrates including plasminogen and insoluble gelatin and type IV collagen.

Addition of physiological concentrations of plasminogen to vesicles shed in serum-free medium increases ^{3}H type IV collagen degradation by vesicles and converts vesicle-associated progelatinases into their active forms, showing that plasmin generated by vesicle-bound uPA activates the gelatinases. Whereas this finding provides further evidence that the gelatinases are located on the vesicle surface, it is also particularly significant in view of the debated role of plasmin in gelatinase activation (64–68). It has recently been shown that treatment of soluble gelatinases with plasmin results in gelatinase degradation. In contrast, addition of physiological concentrations of plasmin(ogen) to uPA- and uPAR-producing cells results in efficient gelatinase activation, showing that gelatinase activation by plasmin requires assembly of all the reactants–uPA, plasmin(ogen), and gelatinases–on the cell surface (46). Thus, gelatinase binding to an insoluble substrate such as the cell or vesicle membrane may protect these enzymes from uncontrolled proteolysis and render them available to partial proteolytic cleavage that results in activation. In addition, vesicles can provide binding sites for uPA and plasminogen and make the activation cascade more efficient. Whether plasmin activates vesicle gelatinases directly or through other proteinases

including MT-MMPs (43–45), MMP-3 (25, 58), or MMP-7 (matrilysin) (69) remains to be investigated. Our data suggest that either plasmin acts directly on vesicle-associated gelatinases or vesicles may also possess MT-MMP, MMP-3, or MMP-7 activities. These hypotheses certainly deserve further investigation.

The presence on the vesicle membrane of a variety of proteolytic enzymes involved in cell invasion, such as the gelatinases and components of the uPA-plasmin system, indicates that these structures are capable of promoting the proteolytic cascade required for the localized degradation of the ECM. Vesicles may provide a large membrane surface for binding of uPA and gelatinases and thus greatly facilitate plasminogen and gelatinase activation. This may represent an important mechanism by which tumor cells degrade the ECM during invasion and metastasis.

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Urokinase Plasminogen Activator and Gelatinases Are Associated with Membrane Vesicles Shed by Human HT1080 Fibrosarcoma Cells

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