ABSTRACT

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that cleave and degrade a wide spectrum of extracellular matrix components. By enhancing turnover of extracellular matrix, MMP activity is also known to play a key role in tumor cell invasion. Because extracellular protease activity requires efficient release of these proteases to the cellular surface, we investigated storage, transport, and exocytosis of MMP-2 and MMP-9 in human melanoma cells using immunofluorescence, electrical, and biochemical techniques. Immunolabeling of melanoma cells with antibodies specific for MMP-2 and MMP-9 led to the identification of two distinct populations of small cytoplasmatic vesicles containing MMP-2 or MMP-9, respectively. In combination with α-tubulin–specific antibodies, both vesicle populations were found to be aligned along the microtubular network. Moreover, the molecular motor protein kinesin is shown to be localized on most of these vesicles, providing evidence that the identified vesicles are actively propelled along microtubules toward the plasma membrane. The functional relevance of these findings is demonstrated using low dosage (5.9 nmol/L) of paclitaxel to affect the microtubular function of melanoma cells. Although cell proliferation is not altered, paclitaxel treatment impairs secretion of MMP-2/MMP-9 and significantly reduces invasive activity in our new cell invasion assay. In conclusion, we demonstrate in melanoma cells that microtubule-dependent trafficking of MMP-containing vesicles and exocytosis are critical steps for invasive behavior and therefore are potential targets for specific antitumor drugs.

INTRODUCTION

Matrix metalloproteinase-2 and matrix metalloproteinase-9 (MMP-2 and MMP-9, also referred as gelatinase A and gelatinase B, respectively) belong to a family of secreted or cell surface–associated zinc-dependent proteases that cleave and disintegrate pericellular substrates. Currently, more than 23 different MMP members have been identified in humans but also in evolutionary distant organisms including invertebrates and plants (1, 2). Each member of the protease family has distinctive but often overlapping specificities for extracellular substrates, such as collagen, elastin, fibronectin, and laminin, and almost any additional extracellular matrix component (3). MMPs are also able to cleave several circulating proteins as well as cell surface proteins (i.e., E-cadherin, fibrin, and interleukin 1; refs. 3 and 4). Thus, MMPs drive the turnover of extracellular matrix proteins and interfere with cell–cell interactions and signaling molecules (5). In normal physiologic situations, activity of MMPs is tightly regulated at the level of transcription, activation of proenzymes by post-translational processing, and inhibition by specific endogenous proteins, i.e., tissue inhibitors of metalloproteinases (TIMPs; refs. 3, 6, and 7). However, at pathophysiologic conditions such as cancer, excessive extracellular MMP activity efficiently breaks down extracellular matrix microstructure (8). Progression of cancer disease is characterized by the formation of metastasis that may arise far away from the primary tumor. Local activity of MMPs influence the tumor microenvironment by destruction and remodeling of the basement membrane barrier and thereby regulate early stages of tumor initiation, growth, and angiogenesis but also invasion and metastasis (4, 9, 10).

Because the ability of cancer cells to invade intact tissue and to form metastasis is directly correlated with the prognosis of the patient, the central role of gelatinases has undergone intensive investigations (4, 11, 12). Several studies have shown that high levels of MMP-2 protease are expressed from different invasive cell lines, such as breast cancer, prostate cancer, or pulmonal cancer (13). Highly aggressive melanoma cells express a significantly higher level of not only MMP-2 and MMP-9, but also MMP-1 and the transmembrane type enzyme MT1-MMP (8, 14). However, invasiveness of tumor cells is rather correlated to the extracellular activity of proteolytic enzymes, especially MMP-2, than to their expression level (3, 15). Most MMPs are released from invading cancer cells and surrounding stromal cells as inactivezymogens that are activated extracellularly (3). After protease secretion, the basic activation of MMPs takes place on the cell membrane surface (16–18). Interestingly, an endogenous TIMP (TIMP-2) participates in MT1-MPP–dependent activation of MMP-2 (19, 20). However, by binding to almost all MMP proteases TIMPs generally inhibit their enzymatic activities (1). Therefore, they have been used in clinical trials as corrective tools to directly affect MMP activity (6).

In fact, several synthetic inhibitors of extracellular MMPs have been developed recently as antitumor drugs (21, 22). Batimastat and marimastat are MMP inhibitors that predominantly block MMP-2 and MMP-9 activity. Batimastat was shown to reduce metastasis of melanoma, mammary carcinoma, and colorectal carcinoma in vitro and in vivo (12). However, because batimastat cannot be administered orally and involves numerous physical side effects, it is no longer tested for clinical trial. Clinical trials using marimastat were disappointing because they failed to increase survival of patients with gastric cancer and advanced pancreatic cancer (4).

The latter approaches attempt to reduce the proteolytic activity of MMPs that is already present in the extracellular tumor environment. In the present study, we focus on intracellular transport mechanisms and secretion of MMP-2 and MMP-9, essential steps that precede extracellular activation of these proteases. We present data demonstrating that human melanoma cells store and transport MMP-2/MMP-9 in small cytoplasmic vesicles that are associated with both microtubules and kinesin, a molecular motor protein. Paclitaxel, a drug that inhibits depolymerization of microtubules, clearly affects secretion of MMPs. Moreover, by using a cell-based invasion assay (23), we show that melanoma cells after exposure to low dosage paclitaxel exhibit reduced invasive capacities.

MATERIALS AND METHODS

Cells and Cell Culture. Highly malignant human amelanotic melanoma cells, subclone A7, are transfected with actin binding protein to maximize their migration activity (24).
Melanoma cells and Madin-Darby canine kidney cells (MDCK–C7 cells) (25) were cultured at standard conditions as described previously (23).

Fluorescence Microscopy. After methanol fixation, cells were double-labeled with primary antibodies (rabbit/mouse anti–MMP-2 and mouse/rabbit anti–MMP-9 serum (Chemicon International, Inc., Temecula, CA), mouse anti-α-tubulin (Sigma, Taufkirchen, Germany), rabbit anti-actin serum (kindly provided by M. McNiven, Mayo Clinic, Rochester, MN)]. After washing, rhodamine red and DTAF-conjugated goat antirabbit/antimouse antibodies were used for secondary labeling (Jackson ImmunoResearch, West Grove, PA). Labeled cells were mounted using Mowiol with added 50 mg/mL 1,4-diazabicyclo-[2.2.2]octane (Sigma-Aldrich, Seelze, Germany). Images were taken using a confocal Fluoview Olympus IX 70 microscope (Olympus, Tokyo, Japan) or a Zeiss Axiosvert 100 microscope (Zeiss, Welwyn Garden City, UK) connected to a CCD camera system (CoolSnap HQ; Photometrics, Tucson, AZ) and analyzed using Meta Morph Imaging system (Universal Imaging, Downingtown, PA).

Western Blotting Analysis. Melanoma cells were cultured for 2 days as described previously (23). Cells inspected to be nearly confluent were washed three times and transferred to serum-free medium. Paclitaxel (5.9 mmol/L) or adenosine 3′(β,γ-imido)triphosphate (AMP-PNP, Sigma-Aldrich; 40 μmol/L) was added. Surprisingly, nonhydrolyzable ATP analog AMP-PNP in concentrations up to 100 μmol/L selectively inhibits ATP-dependent anterograde protein transport along microtubules (26). The cultures were maintained for 22 hours before cell supernatants were harvested and centrifuged twice at 250 × g and 1500 × g. After protein precipitation in an ice-cooled 10% solution of trichloroacetic acid, precipitates were washed in acetone and solubilized in loading buffer. Equal volume samples were separated by denaturing SDS-PAGE (27) and electro-transferred to nitrocellulose membranes as described previously (28). After incubation with antibodies specific for MMP-2 or MMP-9 and peroxidase-conjugated secondary antibodies (Sigma-Aldrich), filters were developed with Supersignal chemiluminescent substrate (Pierce Biotechnology, Rockford, IL).

Gelatin Zymography. Conditioned media were prepared as described for Western immunoblotting. Aliquots of culture media were mixed with equal volumes of sample loading buffer and assays as described previously (29). Gels were stained with Coomassie brilliant blue R250 (Serva, Heidelberg, Germany) to visualize protease activity and were photographed.

Paclitaxel. A stock solution of 5.9 μmol/L paclitaxel (Sigma-Aldrich) in dimethyl sulfoxide (DMSO) was diluted in culture medium to 5.9 μmol/L. For control experiments, equal volumes of DMSO were applied.

Trypan Blue Staining. Vitality and proliferation of paclitaxel-treated melanoma cells within 24 hours were investigated by trypan blue staining (Serva) as described previously (30).

Matrix Metalloproteinase Activity Assay. Melanoma cells were grown on 20-mm glass coverslips to confluence, culture medium was exchanged, and cells were cultured for another 12 hours. MMP activity in the supernatant of control experiments, equal volumes of DMSO were applied.

Statistical Analysis. Mean data of experiments are given ± SE. Statistical significance was tested with unpaired Student’s t test; significant differences of compared values are indicated (P ≤ 0.05).

RESULTS

Matrix Metalloproteinase-2 Is Stored and Transported in Small Cytoplasmatic Vesicles. Human melanoma cells are known to secrete MMP-2 and MMP-9 into the surrounding medium (31). To investigate expression and exocytosis of MMPs in human amelanotic melanoma cells (subclone A7), we used antibodies specific for MMP-2 and fluorescent-labeled secondary antibodies to visualize the intracellular protease by confocal laser scanning microscopy. An overview of typical human melanoma cells is shown in Fig. 1A, exhibiting a pattern of very small punctuated MMP-2–specific signals distributed within the cytoplasm. High magnification imaging unravels that MMP-2–specific staining marks a tremendous number of small vesicular organelles with a diameter of less than 300 nm. In lamellipodia, we identified small numbers of significantly larger structures with a diameter of approximately 0.5 to 1 μm. Surprisingly, MMP-2–positive granules are not randomly distributed throughout the cytoplasm but seemed to be organized in linear configurations. In fact, they are lined up like strings of pearls (Fig. 1B). Shape and distribution of the signals suggest that small MMP-2–containing transport vesicles are associated with the cytoskeleton. To test this assumption melanoma cells were simultaneously labeled with antibodies specific for α-tubulin and MMP-2. Superimposed images confirm a tight intracellular association of these proteins (Fig. 1B–D). In fact, the granular MMP-2 signals are almost perfectly aligned along the microtubular network.

Kinesin is known as molecular motor protein able to actively transport vesicles toward the plus end of microtubules, i.e., toward the plasma membrane (32). To determine whether kinesin is involved in traffic of identified vesicles, we double-stained melanoma cells with both MMP-2–specific and kinesin-specific antibodies. Intracellular kinesin is identified as a dense pattern of punctuated structures that is distributed equally within the cytoplasm of the cells (Fig. 2). The prementioned pattern of small MMP-2 vesicles and kinesin partially overlaps in superimposed images (Fig. 2C). In fact, we found that 81.9 ± 2.3% of analyzed MMP-2–positive granules were colocalized with kinesin-specific granular signals. Furthermore, double stainings of melanoma cells with kinesin- and α-tubulin–specific antibodies also revealed a good correlation of granular kinesin signals with the microtubular network. In summary, these experiments point out that human melanoma cells store MMP-2 intracellularly in small exocytotic vesicles that are most likely directed toward the plasma membrane along microtubules by kinesin.

Intracellular Localization of Matrix Metalloproteinase-9 Is Separated from Matrix Metalloproteinase-2 Storage Vesicles. Because MMP-2 and MMP-9 in many cells are functionally related, we investigated whether both proteases are transported using the same intracellular granules. In double-staining immunofluorescence experiments we observed a comparable appearance and overall distribution of MMP-9 and MMP-2 vesicles. Surprisingly, the gelatinases were found in separate populations of small vesicles that do not colocalize with each other. However, single vesicles containing MMP-2 or MMP-9 were often found in close proximity (Fig. 3A). Staining with antibodies specific for α-tubulin in combination with MMP-9–specific antibodies indicated a tight association of both proteins. Superimposed images display an alignment of the MMP-9–specific granular signals along the microtubular network, most convincingly visualized at the cell lamellipodia (Fig. 3B). Similar to our collo-
ization results concerning MMP-2, we observed that most MMP-9–containing vesicles are also associated with kinesin.

**Paclitaxel Impairs Matrix Metalloproteinase Secretion by Human Melanoma Cells.** Paclitaxel, a common drug that interferes with microtubules, was used as a tool to block microtubular transport function. Paclitaxel binds to the β-tubulin subunit of polymerized tubulin tubes and inhibits depolymerization of those tubes (33). Moreover, it is well known that paclitaxel interferes with the mitotic spindle and therefore inhibits cell proliferation (34, 35). To exclude that the expected effects of paclitaxel on secretion and invasion are due to a difference in cell numbers, proliferation of paclitaxel-treated melanoma cells was measured. After 24 hours of incubation in medium supplemented with 5.9 nmol/L paclitaxel, no significant difference in total cell number could be determined in comparison with control cells. Ninety-one percent of all paclitaxel-treated cells and 87% of control cells were vital (Table 1). Thus, low concentration paclitaxel treatment had no significant effect on A7 melanoma cell proliferation at conditions used in our experiments.

To examine the morphologic changes in melanoma cells caused by exposure to paclitaxel, immunofluorescence techniques were used. After 12 hours of treatment with 5.9 nmol/L paclitaxel in culture medium, we visualized typical bundles of microtubules that were clustered around the cell nucleus of the treated cells. The flattened shape of control cells (Fig. 4A) has changed to a nearly spheroidal appearance with a reduced diameter. No lamellipodia could be observed in drug-treated cells (Fig. 4B). Such alterations characteristic for cells affected by paclitaxel were not observed in control cells. Labeling of paclitaxel-treated melanoma cells with MMP-2–specific antibodies revealed that MMP-2–containing vesicles are still attached to the microtubular system (Fig. 4C), indicating that paclitaxel affects neither vesicle integrity nor vesicle linkage to the microtubular system. Cells allowed to recover for 9 hours after the paclitaxel treatment already start to reconstruct their microtubular organization. Microtubules spread out from the dense bundles around the nucleus forming new networks so that the cell architecture starts to reconstitute. Cell shape appears more flattened with larger diameter, and lamellipodia develop in many cells. These alterations of microtubular constitution indicate that the effects of paclitaxel on the cytoskeleton of melanoma cells are reversible in a rather short time period.

To investigate whether disturbance of microtubular dynamics influences secretion of metalloproteinases, MMP-2 and MMP-9 activity was measured in the supernatant of paclitaxel-treated cells using a gelatinase assay. Indeed, the amount of active enzyme was significantly diminished in the supernatant of melanoma cells with disabled microtubules compared with untreated control cells. This is revealed by a reduced absorbance of the reaction product of the assay kit (ΔOD450 of paclitaxel-treated cells, 132.3 ± 36.0 (n = 11); ΔOD450 of nontreated cells, 240.5 ± 38.5 (n = 9); Fig. 5A). Control measurement using culture medium with 5.9 nmol/L paclitaxel revealed that the presence of paclitaxel does not influence results of the assay kit (data not shown).

To distinguish between different gelatinases and to reveal pro- and active enzyme forms, immunodetections were performed in Western immunoblotting experiments. We collected conditioned media of human melanoma cells untreated or treated with low-dose paclitaxel for 18 hours. Antibodies specific for MMP-2 detect two bands separated at about 72 and 62 kDa, which most likely represent the pro- and active MMP-2 proteases (Fig. 5B). In the presence of paclitaxel, the overall amount of MMP-2 proteins is clearly diminished compared with untreated conditioned media. However, a significant reduction

![Image](https://cancerreres.aacrjournals.org)
was primarily detected for 72-kDa proMMP-2 (Fig. 5B). To a minor degree, the already low amount of 62-kDa active MMP-2 is impaired. Using antibodies specific for MMP-9, we noted a faint immunoreactive protein in the range of pro- and active MMP-9 (92/83 kDa) and a more significant signal at approximately 68 kDa. The latter band most likely represents a further degraded MMP-9 fragment leaving open to question whether it represents an active form of MMP-9. Again, we observed a decrease in the level of the secreted MMP-9 species in response to paclitaxel treatment (Fig. 5C). However, the last mentioned result could not be reproduced in all experiments.

Furthermore, we analyzed MMP activity using gelatin zymography. Medium aliquots produced one gelatinolytic active band corresponding to a protein with an apparent molecular mass of about 66 kDa (Fig. 5D). The enzyme causing this band is identified as proMMP-2, confirmed by comigration with reference samples of osteoarthritic chondrocytes well-established to contain pro- and active MMP-2. In paclitaxel-containing conditioned media, we observed a diminished MMP-2 activity compared with untreated media (Fig. 5D). In addition to proMMP-2, we could not find any additional gelatin degrading activity (i.e., MMP-1, MMP-3, and MMP-9). Moreover, to prove that kinesin is involved in transport of MMP-2–containing vesicles along microtubules, we used the kinesin family inhibitor AMP-PNP. At low concentrations, AMP-PNP is known to inhibit anterograde kinesin-dependent movement (32). Conditioned media of AMP-PNP–treated cells also revealed a reduced overall secretion of MMP-2/MMP-9 proteins and a less prominent gelatinase activity of proMMP-2, as shown by Western immunoblotting and zymography, respectively (Fig. 5B–D). In summary, these findings verify that MMP-2 is the main source of gelatinase activity secreted by human melanoma cells and demonstrate that faultless function of microtubules is critical for regular exocytosis of MMP-2– and MMP-9–containing vesicles.

Melanoma Cells with Reduced Matrix Metalloproteinase Secretion Show Impaired Invasive Properties. Several studies have shown that cancer cells that express high levels of MMPs are characterized by enhanced malignant properties. Therefore, cells with reduced secretion of these MMPs should exhibit less invasiveness compared with cells with an intact traffic machinery. To test this assumption, we used a cell-based invasion assay recently established in our laboratory (23). In contrast to the well-known Boyden chamber, this assay uses an electrically tight epithelial monolayer of MDCK...
cells instead of a reconstituted basement membrane to measure early events of melanoma cell invasion.

For each experiment, 10^5 melanoma cells preincubated with either 5.9 mmol/L paclitaxel (in 0.1% DMSO) or 0.1% DMSO for 12 hours were seeded onto the epithelial MDCK–C7 test monolayer (Fig. 6A). During the following measurement, paclitaxel was absent. TEER was measured in regular intervals (Fig. 6B). Within 24 hours of coculture with untreated melanoma cells, TEER of the MDCK monolayer decreased rapidly to 53.9 ± 5.6% (n = 12) of its initial value. However, paclitaxel-pretreated cells were less aggressive and just affected TEER to 75.1 ± 3.1% (n = 18) of its initial value, indicating that violation of the MDCK monolayer is less pronounced (Fig. 6C).

Fig. 5. Identification of MMP-2/MMP-9 in conditioned media of cultured melanoma cells. A, relative enzyme activity of MMP-2 and MMP-9 released by cultured melanoma cells. Data are shown as ΔOD_{450} (OD_{450} nm[PBS] – OD_{450} nm[MMP sample]) × 10^{-3}. Relative activity of a dilution series of MMP-2–positive control samples is shown on the left; supernatant samples of melanoma cells cultured for 12 hours with or without paclitaxel on the right. B, identification of MMP-2 protein by Western immunoblotting. Serum-free conditioned media were harvested from A7 melanoma cells that were untreated (C), treated with paclitaxel (P), or treated with AMP-PNP (A) as described previously. The results of two independent experiments are shown. Position of molecular mass standards is shown on the left (kDa; top to bottom: 150, 100, 75, 50, and 37). C, MMP-9 protein detected in Western blotting experiments using specific antiserum. Molecular mass standards as shown in B. D, gelatinase zymography of conditioned serum-free media obtained from cells treated with paclitaxel (P) or AMP-PNP (A) or from untreated control cells (C). M, control media without cells. Please note proMMP-2 gelatinase activity, identified by comigration with reference samples containing pro- and active MMP-2. MMP-9 or other gelatinase activity could not be observed.

Fig. 4. Indirect immunofluorescence microscopy of paclitaxel-treated melanoma cells. Cells were treated by paclitaxel for 12 hours before immunostaining using α-tubulin-specific antibodies and confocal imaging. A, untreated control melanoma cell; B, melanoma cell treated with 5.9 nmol/L paclitaxel; please note the rounded cell shape and condensed bundles of microtubules clustered around the nucleus. C, Higher magnification of a paclitaxel-treated melanoma cell indicates that MMP-2–specific vesicles (green) remain colocalized to the microtubular system (red). Scale bar = 20 μm.

Fig. 3. Identification of MMP-2/MMP-9 in conditioned media of cultured melanoma cells. A, relative enzyme activity of MMP-2 and MMP-9 released by cultured melanoma cells. Data are shown as ΔOD_{450} (OD_{450} nm[PBS] – OD_{450} nm[MMP sample]) × 10^{-3}. Relative activity of a dilution series of MMP-2–positive control samples is shown on the left; supernatant samples of melanoma cells cultured for 12 hours with or without paclitaxel on the right. B, identification of MMP-2 protein by Western immunoblotting. Serum-free conditioned media were harvested from A7 melanoma cells that were untreated (C), treated with paclitaxel (P), or treated with AMP-PNP (A) as described previously. The results of two independent experiments are shown. Position of molecular mass standards is shown on the left (kDa; top to bottom: 150, 100, 75, 50, and 37). C, MMP-9 protein detected in Western blotting experiments using specific antiserum. Molecular mass standards as shown in B. D, gelatinase zymography of conditioned serum-free media obtained from cells treated with paclitaxel (P) or AMP-PNP (A) or from untreated control cells (C). M, control media without cells. Please note proMMP-2 gelatinase activity, identified by comigration with reference samples containing pro- and active MMP-2. MMP-9 or other gelatinase activity could not be observed.

DISCUSSION

We have recently shown that MMP-2 and MMP-9 activity plays a pivotal role in melanoma cell invasion (31). In the present study, we investigated the mechanism of intracellular transport and exocytosis of MMPs in human melanoma cells. We localized intracellular MMP-2 and MMP-9 stored and transported in small cytoplasmic vesicles. Although similarly distributed in the cytoplasm, these proteases are not colocalized with each other. Vesicular organelles of both MMP-2 and MMP-9 are aligned along microtubules and therefore are likely to be intended for exocytosis at the plasma membrane. A well-known ATPase motor protein that powers vesicular transport along microtubules is kinesin (36, 37). Immunofluorescence stainings revealed that kinesin is localized to ~80% of MMP-2–containing structures. This indicates that the majority of identified organelles are indeed exocytotic vesicles moving to the plasma membrane (38). On the other hand, a small number of MMP-2 vesicles were not found to be associated with kinesin. These may represent vesicles that move into retrograde direction toward the minus end of microtubules. Alternatively, these vesicles may have reached the plasma membrane or may be associated with the submembrane cytoskeletal actin network. Not surprisingly, we also identified punctuated kinesin-specific labeling inside melanoma cells not colocalized with MMP-2 vesicles (about 40% of total kinesin-specific signals). This population is probably involved in transport of vesicles that do not contain MMP-2 but different protein cargo, for instance other members of the MMP family. In fact, we observed that kinesin is also associated with MMP-9–containing vesicles. Besides this, part of the non-colocaliz-
Fig. 6. Invasive activity of A7 tumor cells is affected by paclitaxel. A, schematic model showing the experimental setup of the cell-based assay using filter cups. Cancer cells (A7) seeded on top of a tight MDCK-C7 monolayer reduce TEER. B, time course of single TEER experiments without melanoma cells (A; △), after addition of paclitaxel-pretreated melanoma cells (B; ●), and after addition of untreated melanoma cells (C; □). C, TEER experiments using melanoma cells pretreated for 12 hours with paclitaxel exhibit reduced invasive activity, shown after 24 hours of coculture, compared with untreated melanoma cells and to experiments without addition of melanoma cells. Number of experiments is given in brackets, and SE is displayed.

...ing population may represent a cytoplasmic pool of free kinesin that is not involved in vesicle transport (32).

The data presented thus far disclose that intracellularly MMP-2 and MMP-9 are stored in vesicles that are linked to microtubules. Therefore, disruption of the microtubular system should inhibit transport of both MMPs to the plasma membrane and perturb the delivery of the proteases. Several antimicrotubular drugs such as colchicine and Vinca alkaloids are used in treatment of malignant tumors to date (39). One of these drugs that binds to polymeric tubulin and thereby interferes with the microtubular system is paclitaxel (33). Paclitaxel inhibits disassembly of microtubules, whereas most other known antimicrotubule drugs inhibit microtubule assembly. Paclitaxel is also known to inhibit cell growth, proliferation, and cell motility and is used as a first and second line therapy for patients suffering from cancer (40–42). Parekh and Simpkins (35) reported that half maximal inhibition of proliferation in different rodent tumor cell lines occurred at concentrations of >100 nmol/L, whereas comparable effects in human tumor cells (ovarian carcinoma cells) occurred already at concentrations <10 nmol/L. In human prostate cancer cell lines, paclitaxel concentrations of more than 100 nmol/L were needed to observe microtubular bundling, which was efficient only at a concentration of 500 nmol/L (43). During therapy, even cases of paclitaxel-resistant cells were described previously (44). Because of this differential response of human cell lines to paclitaxel, we investigated proliferation of human amelanotic melanoma cells after 24 hours of paclitaxel incubation. No significant inhibition of proliferation in cells treated with 5.9 nmol/L paclitaxel could be observed. However, as proved by immunofluorescence imaging, short-time (12 hours) paclitaxel treatment had substantial influence on the microtubular organization. Paclitaxel-treated cells exhibited condensed microtubule bundles around the nucleus, and lamellipodia were missing. We additionally could show that this effect of paclitaxel on microtubules is reversible within a time period of 9 hours. In paclitaxel-treated cells MMP-2-containing vesicles remained colocalized to the cytoskeleton, suggesting that paclitaxel does not interfere with vesicle binding to microtubules. Nevertheless, paclitaxel leads to dysfunctions in vesicular transport. Indeed, release of vesicular content of melanoma cells was hindered as was shown by a reduced amount of active MMP-2 in the supernatant of paclitaxel-treated melanoma cells using a gelatinolytic assay kit, zymography, and Western blotting. We exclude that a decreased cell number caused this depletion of MMPs, because under conditions used in our experiments we observed no significant inhibition of cell proliferation. It is also worth mentioning that the presence of paclitaxel predominantly results in a diminished amount of the pro-form of MMP-2 and to a lesser degree in reduced activated MMP-2. Thus, paclitaxel lowers the level of MMP-2 secretion, and it appears not to accelerate processing/activation of proMMP-2 at our experimental conditions. Stein and Wang (43, 45) have shown that paclitaxel blocks MMP-2 production and secretion in PC-3ML cells in vitro and in established bone tumors but does not seem to affect MMP inhibitor TIMP-1 or TIMP-2 level. With human melanoma cells, we did rather note a slight decrease in the secretion of TIMP-2 during low-dose paclitaxel treatment (data not shown). However, treatment of other cancer cell types with higher dosage of paclitaxel (46) or microtubule-disrupting drugs (47) displayed controversial results.

Recently, we established a cell-based invasion assay based on a confluent epithelial cell monolayer as a test barrier for invasive cells. The assay is able to monitor very early steps of cell layer destruction by cancer cells independent of physical contact between tumor and epithelial cells (31). The ability of melanoma cells to invade into the intact cell layer is based on the extracellular activity of locally secreted proteases, especially MMPs (23, 31, 48). In the current study, this assay was used to investigate the impact of microtubule-dependent protease secretion on cancer cell invasion. Melanoma cells were predrugged for a period of 12 hours using less than 6 nmol/L paclitaxel and were afterward applied to the test system. During coculture, the TEER breakdown was attenuated by almost 50%, compared with control melanoma cells. Because paclitaxel was not present during the invasion experiments and the microtubular organization was shown to reconstitute already 9 hours after completion of drug treatment, a significant reduction of melanoma cell invasiveness was particularly evident during the first hours of the experiments but still obvious after 24 hours. However, time delay of melanoma cell invasion might have been even more evident if paclitaxel had been present during invasion experiments. Notwithstanding, it is possible that release of other proteases may also be inhibited as a consequence of this drug treatment. However, several lines of evidence suggest that primarily the reduced release of MMP-2 and MMP-9 is responsible for the diminished invasion activity. First, in conditioned media, we could not detect any other gelatinase activity (i.e., MMP-1, MMP-3, and MMP-9) except MMP-2 using zymography experiments. Second, in immunoblotting experiments, both gelatinases were identified in cell supernatants and depleted in response to paclitaxel. Third, in former experiments ilomastat, a specific inhibitor for MMP-1, -2, -3, -8, and -9, but not pepstatin A and leupeptin, was shown to inhibit invasion activity of these melanoma cells (31). Moreover, using purified collagenase A instead of melanoma cells, we confirmed that MMP activity is critical for resistance breakdown in our assay system (31). Fourth, because we demonstrated that physical contact is not necessary to induce resistance breakdown, it is very unlikely that changes...
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REFERENCES


MMP-2/MMP-9 Transport in Melanoma Cells

In the article on MMP-2/MMP-9 transport in melanoma cells in the December 15, 2004 issue of Cancer Research (1), the label on the ordinate of Fig. 1C was incorrect. The label on the ordinate should have read “TEER \((t = 24 \, \text{h})/\text{TEER} \,(t = 0 \, \text{h}).\)"

Microtubule-Dependent Matrix Metalloproteinase-2/Matrix Metalloproteinase-9 Exocytosis: Prerequisite in Human Melanoma Cell Invasion

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