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Inhibition of Hyaluronan Export Attenuates Cell Migration and Metastasis of Human Melanoma

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ABSTRACT

When secreted from malignant cells, hyaluronan facilitates tumor invasion and metastasis, as inhibition of its export by zaprinast inhibited metastasis formation in mice. However, the precise steps of the metastatic cascade, which were influenced by zaprinast, have not been identified as yet. Here we analyzed the cell biological effects of the inhibitor on three human melanoma cell lines that differed in their hyaluronan production and their metastatic capability when xenografted into SCID mice. We measured the influence of zaprinast on cellular hyaluronan export, surface coat formation, proliferation, random migration, colony formation in soft agar, adhesion, and transepithelial resistance. Concentrations of zaprinast not affecting cell proliferation, adhesion and transepithelial resistance, nevertheless reduced hyaluronan export by 50%, surface coat formation, random migration, and colony formation in soft agar. These results indicate that hyaluronan enhances metastasis formation primarily in those steps of the metastatic cascade, which involves tumor cell migration. J. Cell. Biochem. 105: 1260–1266, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: HYALURONAN EXPORT INHIBITION; METASTASIS; CELL MIGRATION; INVASION; PROLIFERATION; CELL ADHESION

Several cellular processes are required for successful metastasis formation, including cell proliferation at the site of the primary tumor, loosening of the future metastatic cell from the local tumor, degradation of the local stroma, intravasation into the lymphatic or blood vessels, survival within the circulation, extravasation at the site of the future metastasis, and finally initiation and maintenance of growth at the secondary site [Chambers et al., 2002]. Many of these processes involve cell–cell or cell–matrix interactions, both processes affected by hyaluronan [Toole, 2004]. For example, mouse mammary carcinoma cell lines selected for low levels of hyaluronan production were shown to give rise to fewer disseminated nodules in the lung after intravascular injection than lines that produced high levels of hyaluronan [Itano et al., 1999] indicating that hyaluronan expression is involved in tumor cell dissemination. Because of the close association of high hyaluronan levels with malignancy in many tumor types observed in clinical samples, several groups have used experimental manipulations in animal models to test which step of the metastatic cascade is influenced by hyaluronan. Increased hyaluronan production, which was induced by transfection with cDNAs encoding the three hyaluronan synthases *HAS*1, *HAS*2 or *HAS*3, caused increased growth and/or metastatic potential of tumors in xenograft models of fibrosarcoma and prostate, colon and breast cancer [Itano et al., 1999; Kosaki et al., 1999; Li and Heldin, 2001; Liu et al., 2001; Jacobson et al., 2002]. Correspondingly, reduction of hyaluronan production in prostate carcinoma cells using *HAS* antisense mRNAs caused decreased tumor growth [Simpson et al., 2002; Itano et al., 2004; Nishida et al., 2005; Udabage et al., 2005a,b; Li et al., 2007]. An inhibitor of hyaluronan production, 4-methylumbelliferone, inhibited B16-F10 melanoma cell adhesion, locomotion, and liver metastasis [Kudo et al., 2004; Yoshihara et al., 2005; Nakazawa et al., 2006].

The biosynthesis of hyaluronan and proteoglycans have different mechanisms and occur in different compartments of the cell [Prehm,

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1984]. Hyaluronan is polymerized at the inner side of plasma membranes [Prehm, 1983a,b, 1984, 2006]. Recently, we discovered that a variety of multidrug resistance inhibitors interfered with hyaluronan export by the multidrug resistance associated protein MRP5 [Prehm and Schumacher, 2004; Schulz et al., 2007]. Some of the hyaluronan export inhibitors were already applied to prevent hyaluronan overproduction and proteoglycan loss in Il-1α activated chondrocyte cell cultures, cartilage organ cultures and in an animal model of osteoarthrosis in vivo and in vitro [Prehm, 2005].

Furthermore, we characterized human melanoma cells with regard to their growth and metastatic pattern in a SCID mouse xenograft model [Thies et al., 2007] and now extended our studies to identify those cellular reactions in which hyaluronan synthesis contributes towards metastasis formation using recently the discovered inhibitor of hyaluronan export zaprinast [Prehm and Schumacher, 2004; Schulz et al., 2007; Deiters and Prehm, 2008]. Zaprinast is a structural analogue of cGMP that inhibits the cGMP specific phosphodiesterase (PDE5) in nanomolar concentrations [Kulkarni and Patil, 2004]. It has been shown to reduce the malignancy of certain tumors in the absence of any cytotoxic drugs [Drees et al., 1993; Giorgi et al., 2001]. However, the ability of zaprinast to block hyaluronan export was unknown at that time. Since the underlying mechanism of malignancy reduction remained unknown, we here utilized zaprinast to analyze the effects of reduced hyaluronan synthesis on cellular behavior occurring in the different steps of the metastatic cascade.

MATERIALS AND METHODS

CELL CULTURE

Details of the cell lines LOX, Mel6, and MeWo were described by Thies et al. [2007]. Cells were grown in suspension culture in Dulbecco's Modified Eagles medium supplemented with streptomycin/penicillin (100 units of each/ml), kanamycin (100 units/ml), and 10% fetal calf serum. The formation of cell surface hyaluronan by a particle-exclusion assay as described [Goldberg and Toole, 1984].

DETERMINATION OF HYALURONAN EXPORT

The cells were incubated for 24 h at 37°C, the media were replace with fresh media and after additional 24 h aliquots (5 and 20 μl) of the culture medium were used for measurement of the hyaluronan concentration in the cell culture medium by an ELISA. The wells of a 96 well Covalink-NH-microtiter plate (NUNC) were coated with 100 μl of a mixture of 100 μg/ml of hyaluronan (Healon®), 9.2 μg/ml of *N*-Hydroxysuccin-imide-3-sulfonic acid and 615 μl/ml of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide for 2 h at room temperature and overnight at 4°C. The wells were washed three times with 2 M NaCl, 41 mM $MgSO_4$, 0.05% Tween-20 in 50 mM phosphate buffered saline pH 7.2 (buffer A) and once with 2 M NaCl, 41 mM $MgSO_4$, in phosphate buffered saline pH 7.2. Additional binding sites were blocked by incubation with 300 μl of 0.5% bovine serum albumin in phosphate buffered saline for 30 min at 37°C. Calibration of the assay was performed with standard concentrations of hyaluronan ranging from 15 to 6,000 ng/ml in equal volumes of culture medium as used for measurement of the cellular supernatants. A solution (50 μl) of the biotinylated hyaluronan binding fragment of aggrecan (Applied Bioligands Corporation, Winnipeg, Canada) in 1.5 M NaCl, 0.3 M guanidinium hydrochloride, 0.08% bovine serum albumin, 0.02% NaN_3, 25 mM phosphate buffer pH 7.0 was pre-incubated with 50 μl of the standard hyaluronan solutions or cellular supernatants for 1 h at 37°C. The mixtures were transferred to the hyaluronan-coated test plate and incubated for 1 h at 37°C. The microtiter plate was washed three times with buffer A and incubated with 100 μl/well of a solution of streptavidin-horseraddish-peroxidase (Amersham) at a dilution of 1:100 in phosphate buffered saline, 0.1% Tween-20 for 30 min at room temperature. The plate was washed five times with buffer A and the color was developed by incubation with a 100 μl/well of a solution of 5 mg *o*-phenylenediamine and 5 μl 30% H_2O_2 in 10 ml of 0.1 M citrate-phosphate buffer pH 5.3 for 25 min at room temperature. The adsorption was read at 490 nm. The concentrations in the samples were calculated from a logarithmic regression curve of the hyaluronan standard solutions.

DETERMINATION OF CELL MIGRATION AND PROLIFERATION

These assay were performed as described previously [Dube et al., 2001]. Briefly, the cells were seeded at a density of 1×10^6/35 mm on uncoated culture dishes and grown to confluency. A scratch was made within the monolayer with a sterile Pasteur pipette tip. The same spot was photographed under phase contrast microscopy at various time intervals. The migrated distance was assessed using 16 measurements per time point and cell line in four independent experiments. The growth rate was determined by cell counting after trypsinization at various time points after seeding.

DETERMINATION OF CELL ADHESION

For the cell adhesion assay, LOX cells were labeled with CFSE (Carboxy Fluorescein Succimidyl Ester) [Gruber et al., 2000]. Cells were transferred into 96 well microtiter plates at a density of 4×10^4/well and incubated for various times at 37°C. Medium was withdrawn and the cells were washed with phosphate buffered saline and the fluorescence was determined by a fluorescent microtiter plate reader.

COLONY FORMATION IN SOFT AGAR

Colony formation in soft agar was measured as described by Ghatak et al. [2002]. Briefly, the assay was performed in 6-well plates with a basal agar layer containing 0.6% agar in DMEM, 10% fetal bovine serum. This layer was overlaid with a second layer of 1 ml of 0.2% agar containing 20% fetal bovine serum, mixed with 1 ml of a suspension of 25,000 cells, with or without addition of zaprinast. The plates were incubated at 37 °C for 10–14 days, and the diameter of tumor colonies was determined with a microscope.

MELANOMA CELL INVASION ASSAY

The invasive properties of melanoma cells were investigated by a cell-based assay. An epithelial MDCK–C7 cell monolayer on a thin filter membrane (growth area, 4.2 cm^2; pore diameter, 0.4 μm; thickness, 20 μm; Falcon, Heidelberg, Germany) served as a test barrier for the invasive capabilities of melanoma cells. When confluent, these cells from a tight epithelial sheet with a high

TABLE I. Hyaluronan Production and Metastatic Properties of Human Melanoma Cell Lines Xenografted Into SCID Mice

Cell line	Hyaluronan in cell culture (ng/ml)	Tumor wet weight (g)	Metastases (number)	Metastasis rate (%)	Growth period (days)
LOX	390	1.56	168	100	25
Mel6	5	0.89	24,031	100	90
MeWo	0	0.69	2,860	60	90

The metastasis rate is the percentage of animals that develop metastases after inoculation with the cell lines.

trans-epithelial electrical resistance (TEER), which was measured continuously using a STX-2 electrode (WPI, Sarasota). Permeabilization of the epithelial cell layer (MDCK-C7 cells) due to the invasive activity of the melanoma cells can be determined by TEER measurements, as previously reported [Ludwig et al., 2002; Schnaeker et al., 2004]. Melanoma cells were added to the MDCK monolayer after TEER had reached a value of about 8,000 $\Omega\,cm^2$. The time point of melanoma cell addition was defined as 0 h. Culturing of cells was performed as described above. A resistance breakdown due to the invasive activity of melanoma cells occurred between 20 and 45 h in relation to time point 0 h.

RESULTS

CHARACTERIZATION OF METASTATIC MELANOMA CELL LINES

To analyze the effect of hyaluronan on individual steps of the metastatic cascade, we chose three human melanoma cell lines that differed markedly in their metastatic potential and tumor weight in the SCID mouse model (Table I) [Thies et al., 2007] and compared these properties with their content of hyaluronan in cell culture. The cell line LOX produced large amounts of hyaluronan in cell culture and produced so large tumors that the animals had to be sacrificed after 25 days. The cell line Mel6 produced low amounts of hyaluronan in cell culture and MeWo did not produce any hyaluronan. The number of metastases could not be compared directly at day 25, because Mel6 and MeWo tumors had not developed after 25 days and had to be determined after 90 days. From the data it is evident that the LOX cell line had the highest metastatic potential. Table I also shows a clear correlation between the cellular hyaluronan production in vitro and the weight of primary tumors grown in SCID mice.

INFLUENCE OF ZAPRINAST ON HYALURONAN EXPORT

Initially we determined, whether zaprinast indeed reduced the hyaluronan export in the hyaluronan producing cell lines LOX and Mel6. Figure 1 shows that zaprinast reduced hyaluronan export in LOX cells to about 60% at concentrations between 10 and 50 μM. In Mel6 cells, hyaluronan export was reduced even further starting at 6 μM and was lower than 20% at 100 μM. Zaprinast did not have any effect on the hyaluronan synthase activity at these concentrations (data not shown).

PROLIFERATION

We have shown previously that zaprinast inhibited hyaluronan export and proliferation of skin fibroblast in a concentration dependent manner [Prehm and Schumacher, 2004; Schulz et al., 2007]. It was therefore of interest to examine these effects of zaprinast on melanoma cells as well. Figure 2 shows that LOX cells proliferated almost twice as fast as the other two cell lines. Growth inhibition of the three cell lines started at concentrations above 25 μM of zaprinast. Thus, inhibition of hyaluronan synthesis and export below 25 μM cannot be attributed to cytotoxic effects.

HYALURONAN COAT FORMATION

If zaprinast inhibited hyaluronan export, it should also reduce hyaluronan coat formation. We examined this by a particle-exclusion assay [Goldberg and Toole, 1984] and the effects of zaprinast upon LOX are illustrated in Figure 3. When the cells were incubated without zaprinast, a distinct pericellular halos were visible and these were significantly reduced by zaprinast. For quantitative estimation of the surface area, we determined the average the number of erythrocytes around the cells being 48 for the controls and 30 for the zaprinast treated cells, respectively. This results in a reduction of the occupied surface area by about 60%.

CELL MIGRATION AND INVASION

Cell migration was determined by the scratch assay [Dube et al., 2001]. Figure 4 shows that zaprinast reduced cell migration already at concentrations of 12.5 μM, when no significant effect on proliferation was observed. LOX cells that produced the highest

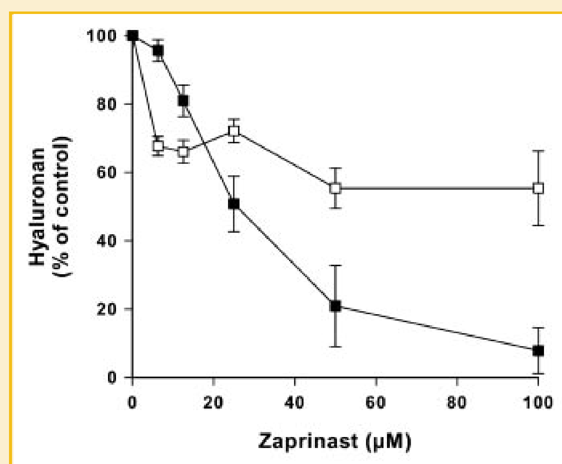

Fig. 1. Inhibition of hyaluronan export from the human melanoma cell lines LOX (□) or Mel6 (■) by zaprinast. Subconfluent cells were grown with increasing concentrations of zaprinast for 2 days. The medium was changed after the first day and used for the determination of hyaluronan on the second day. The error bars indicated SD of three determinations.

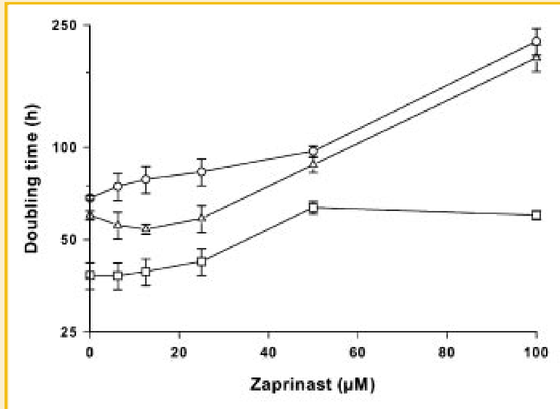

Fig. 2. Proliferation of the human melanoma cell lines LOX (□), Mel6 (○), or MeWo (△) in the presence of increasing concentrations of zaprinast. Subconfluent cells were grown with increasing concentrations of zaprinast. After 1, 2, 3, and 4 days, the cells were detached by trypsin and their number was determined by a Cell Counter. The error bars indicated SD of three determinations.

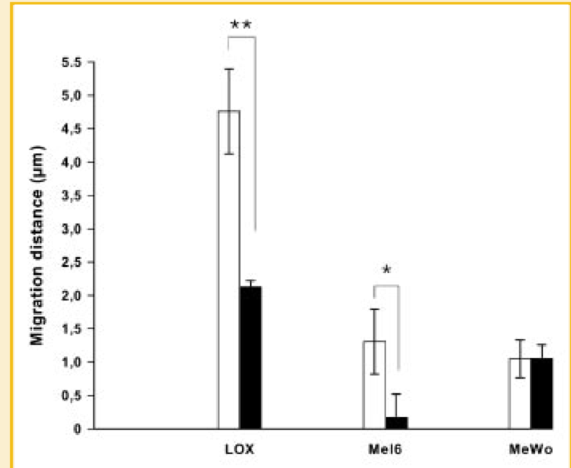

Fig. 4. Cell migration in the absence (open bars) or presence of 12.5 µM of zaprinast (solid bars). Cells were grown to confluency and a scratch of 6.25 µm in diameter was applied. Migration of the cells into the wound was documented by a camera and the distance of wound closure was determined at different time intervals. The error bars indicated SD of eight determinations after 10 h of cultivation. $^{**}P < 0.0001$, $^{*}P < 0.001$ (unpaired Student's *t*-test).

amount of hyaluronan migrated the longest distance. In both hyaluronan producing cell lines, the migration rate could be reduced by inhibition of hyaluronan export with zaprinast.

COLONY FORMATION IN SOFT AGAR

A hallmark characteristic of tumor cells, however, is their ability to grow in an anchorage-independent manner [Kosaki et al., 1999; Ghatak et al., 2002]. The hyaluronan export inhibitor zaprinast decreased anchorage-independent growth of tumor cells, as assayed by the ability to grow as colonies in soft agar (Fig. 5).

CELL ADHESION

The kinetics of cell adhesion to polystyrene tissue culture dishes was determined first. Figure 6 shows that the cell lines differed in the adhesion rates, but inhibition of hyaluronan export had no significant influence on the adhesion rate of each individual cell line, even when zaprinast was applied at a concentration of 50 µM (data not shown).

We also determined the adhesion of LOX cells to fibroblasts or endothelial cells. Fewer than 5% of the LOX cells adhered to either fibroblasts or endothelial cells and the adherence rate could not be increased by the hyaluronan export inhibitor zaprinast.

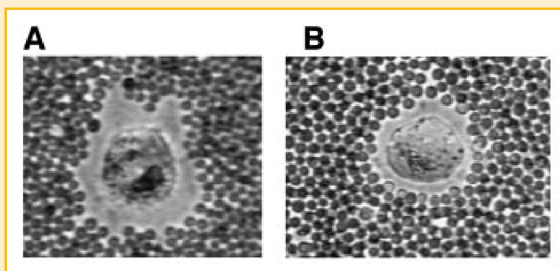

Fig. 3. Cell surface hyaluronan coat. Growing LOX cells were incubated for 1 day in the absence (A) or presence (B) of 50 µM zaprinast. A suspension of red blood cells was settled onto the cells and images were taken after 2 h. The surface area occupied by the cells coats was reduced by about 60% by zaprinast. At a zaprinast concentration of 6.25 µM the area was reduced by about 50% (data not shown).

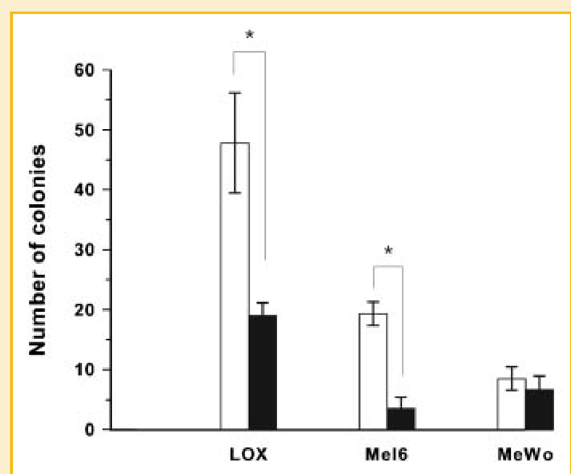

Fig. 5. Colony formation in soft agar in the absence (open bars) or presence of 12.5 µM of zaprinast (solid bars). Cells were plated in soft agar at ~25,000 cells per well of 6-well plates and the cultures were incubated for 14 days at 37°C. The numbers of colonies larger than 0.2 mm in size were counted. The error bars indicated SD of six determinations. $^{*}P < 0.001$ (unpaired Student's *t*-test).

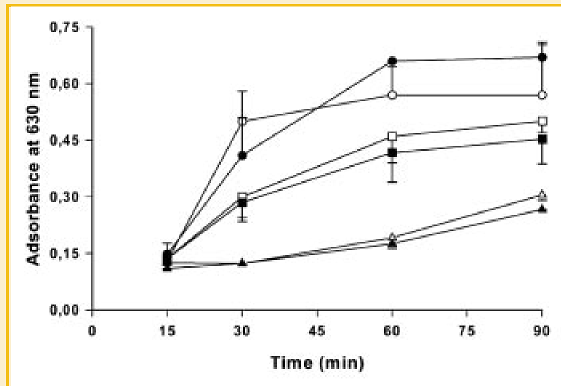

Fig. 6. Adhesion of the human melanoma cell lines LOX (□,■), Mel6 (○,●), or MeWo (△,▲) in the absence (open symbols) or presence (solid symbols) of 12.5 μM of zaprinast. The cells were seeded onto 2 cm^2 plastic dishes and at the times indicated cell adhesion was determined by measuring the amount of protein that remained on the dish after washing off non-adherent cells. The error bars indicate the SD of three independent experiments.

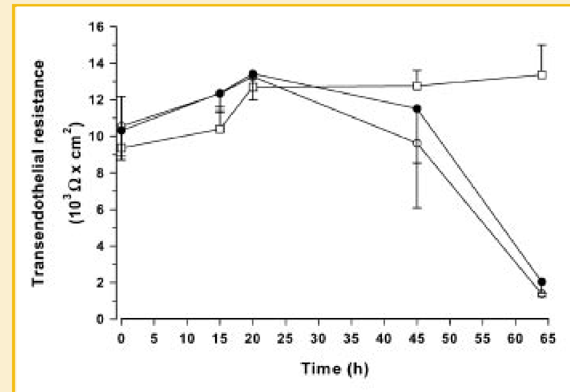

Fig. 7. Breakdown of electrical resistance of an epithelial cell monolayer. Mel6 (○,●) cells were seeded in the upper medium on the filter membrane at day 1 in the absence (open symbols) or presence (solid symbols) of 12.5 μM of zaprinast. Control cultures did not receive any melanoma cells (□). Transepithelial electrical resistance was measured at daily time intervals. The error bars indicate the SD of six determinations.

TRANSEPITHELIAL MIGRATION

Because invasion in vivo also involves transepithelial migration, we used the transepithelial cell invasion assay that is performed using a modified Boyden chamber system [Ludwig et al., 2002; Schnaeker et al., 2004]. A tight epithelial monolayer of MDCK-C7 was grown to confluence on a polycarbonate membrane with a defined pore size of 0.4 μM. Since the LOX cell line did not adhere to epithelial monolayers, we performed this experiment with the Mel6 cell line. Mel6 melanoma cells were added to the upper chamber and incubated for 24 h in the presence and absence of zaprinast. Invading cells produce a loss of integrity of the monolayer at first followed by holes in the epithelial layer. Both steps of invasion activity are reflected by a decrease of the electrical resistance. The effect of hyaluronan export inhibition was performed with Mel6 cells. Figure 7 shows that hyaluronan export inhibition by addition of zaprinast (100 μM) did not have any significant effect on transepithelial electric resistance. Similar results were obtained with the hyaluronan producing melanoma cell line A7 [Kochanowski et al., 2006] (data not shown).

DISCUSSION

The present experiments were performed to investigate the influence of the multidrug resistance and phosphodiesterase inhibitor zaprinast, which also suppresses the export of newly synthesized hyaluronan through the plasma membrane on cellular processes essential for the metastatic spread of malignant tumors. We focused on hyaluronan, as hyaluronan overproduction in malignant tumor tissues is associated with a poor prognosis resulting from the higher metastatic potential of the hyaluronan producing tumor cells [Itano et al., 1999; Kosaki et al., 1999]. One explanation for this higher metastatic rate has been found in transfection experiments, where the motility of malignant tumor cells, which is one important biological process for increased metastatic capability, was enhanced by transfection with a hyaluronan synthase expression construct [Ichikawa et al., 1999]. As hyaluronan export mediated by MRP5 can be inhibited by MRP5 multidrug resistance inhibitor zaprinast [Prehm and Schumacher, 2004; Schulz et al., 2007], we treated three human melanoma cell lines with zaprinast to analyze which cellular reactions in the multi step metastatic process could be attenuated by zaprinast. Zaprinast was chosen, because it reduced metastasis formation in mice albeit in other malignancies than melanoma [Drees et al., 1993; Giorgi et al., 2001]. However, the biochemical reason for this biological anti-metastatic effect remained elusive at the time.

Zaprinast inhibited hyaluronan export from the hyaluronan producing melanoma cell lines in a concentration dependent manner. At concentrations up to 25 μM, zaprinast reduced hyaluronan export significantly without inhibiting cell proliferation corroborating earlier findings in synovial fibroblasts, where a similar independence of cell proliferation and hyaluronan production could be demonstrated [Prehm and Schumacher, 2004]. This independence of hyaluronan production from proliferation is in contrast to normal skin fibroblasts and hyaluronan synthase transfected CHO cells, both cells in which cell proliferation and mitosis depends on hyaluronan synthesis [Brecht et al., 1986; Dube et al., 2001]. It is also in contrast to the metastatic neuroblastoma cell lines and HT-29 colon cancer cells whose proliferation in cell culture was inhibited by zaprinast [Drees et al., 1993; Giorgi et al., 2001]. Thus the dependence of mitosis and cell proliferation on hyaluronan synthesis is particular to the cell type and not generally related to tumor metastasis. This in turn implicates that proliferation and metastasis are not directly coupled.

Nevertheless, it is interesting to compare the metastatic properties of the three human melanoma cell lines with the amounts of hyaluronan produced and the proliferation rates in cell culture (Table I). LOX cells produced the largest tumors in vivo, and proliferated and migrated fastest and produced most hyaluronan in

vitro. The LOX cells were so fast growing that the mice had to be sacrificed after 25 days, at a time point when Mel6 or MeWo tumors were not yet or barely detectable. In vivo LOX cells thus had the highest metastatic potential indicating that increased hyaluronan synthesis in metastatic tumors correlated with cell proliferation and subsequently tumor size, but not with the number of metastasis.

We demonstrated here that the hyaluronan export inhibitor zaprinast also reduced the sizes of the cell surface hyaluronan coats, cell migration and colony formation in soft agar. Cell migration and colony formation in soft agar was particularly sensitive to reduction of hyaluronan export, as low concentrations of zaprinast (12.5 μM) reduced hyaluronan export by only about 20%, while cell proliferation, adhesion, and transepithelial resistance was only insignificantly affected.

From the above considerations, we can summarize the following facts. (1) Metastasis is dependent on migration. (2) Hyaluronan production enhances metastasis and migration. (3) The hyaluronan export inhibitor zaprinast inhibited metastasis, hyaluronan export and migration in the hyaluronan producing cells LOX and Mel6, but did not inhibit migration in hyaluronan deficient cells MeWo, indicating that the effect on cell migration was specific and not attributed to other cellular effects of zaprinast. Taken together, these facts indicate that the augmenting influence of hyaluronan on metastasis formation is primarily through migration and not on the other parameters tested such as cell adhesion and proliferation.

The main mechanism of hyaluronan synthesis on migration and metastasis could be its effect on shedding from the cell membrane. Shedding of hyaluronan can also be influenced by the hyaluronan receptor CD44. We showed previously that CD44 and its proteolysis determines synthesis and shedding of hyaluronan from the cell surface the metastatic potential of melanoma cells [Lüke and Prehm, 1999]. Thus the metastatic potential is also dependent on cell surface proteases that indirectly regulate hyaluronan synthesis through CD44.

All hyaluronan export inhibitors known up to now have been developed for other purposes and are certainly not ideal for treatment of metastasis. It may be highly desirable to develop specific hyaluronan export inhibitors, because they could not only be applied in tumor treatment [Adamia et al., 2005], but also for many other diseases that are associated with hyaluronan overproduction.

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