Increased invasive potential and up-regulation of MMP-2 in MDA-MB-231 breast cancer cells expressing the ß3 integrin subunit

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Abstract. Integrins are a family of transmembrane adhesion receptors that might transduce signals from the extracellular matrix into the inside of cells after ligand binding. In order to investigate whether ß3 integrins expressed in tumor cells might mediate such outside-in signaling, human MDA-MB-231 breast cancer cells that were stably transfected with either ß3 integrin or mock-transfected were investigated in a matrigel degradation assay and a grafting experiment was performed on the developing chicken chorioallantoic membrane (CAM). After cultivation on matrigel for time periods between one and five days, more matrigel was digested in the wells in which ß3 integrin expressing cells were incubated than in wells of mock-transfected cells. Furthermore, extracts of ß3 integrin expressing cells contained higher levels of MMP-2 protein as determined by immunoblotting and more MMP-2 associated gelatinase activity as detected by zymography than extracts of mock-transfected cells. Matrigel degradation and gelatinase activity as well as MMP-2 expression were elevated when ß3 integrin expressing cells were incubated in the presence of the RGD peptide (mimicking an integrin ligand). After grafting on 10 day-old embryonic chicken CAM for three to five days, ß3 integrin expressing cells assembled in spheroids showed higher rates of spreading on the CAM surface and CAM invasion as well as a significant MMP-2 up-regulation compared to mock-transfected cells. The results from the *in vivo* and *in vitro* experiments allow the conclusion that the presence of ß3 integrin in MDA-MB-231 breast cancer cells induced an increased MMP-2 expression and activity that might contribute to the enhanced invasive potential observed.

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Introduction

Integrins are a family of transmembrane cell-surface receptors participating in cell-to-cell and cell-to-extracellular matrix (ECM) interactions. Functionally active integrins are composed of α- and ß-subunits. Hitherto, 24 integrin heterodimers with distinct cellular and adhesive specificities have been characterized (1,2). Furthermore, about 20 different signaling proteins might co-operatively associate with integrins initiating signaling cascades which are involved in cell differentiation, adhesion, migration, invasion, proliferation, angiogenesis and survival (3,4). Several of these signaling cascades involve ras or phosphatidyl inositol-3-kinase, and have been demonstrated to prevent apoptosis (5,6).

Among the 18α integrin subunits, only the αv and αIIb subunits might interact with the ß3 subunit to assemble as αvß3 and αIIb ß3 integrins, respectively. The αvß3 integrin that belongs to the most actively investigated members of the integrin family binds to various ECM and soluble ligands, e.g. vitronectin, osteopontin, thrombospondin-1 and matrix metalloproteinase (MMP)-2. Under non-pathological conditions this integrin is found in blood vessels while αIIb ß3 integrin with binding specificity for vitronectin and prothrombin is expressed primarily within cells of the megakaryocyte lineage (7).

Performing immunohistochemistry, αIIb ß3 integrin was detected in a variety of tumor cells in different transformed organs/tissues, such as cancers of lung, liver, kidney, colon, bladder, breast, prostate and cervix (8). In ovarian tumors, the appearance of αvß3 correlated with the grade of differentiation (9). In accordance with the localization patterns it was suggested that both ß3 integrins are involved in tumor progression (10-12) and participate in organ-targeting and subsequent invasion of tumor cells (reviewed in ref. 13). Such a correlation between αvß3 integrin expression and tumor progression and/or invasiveness also has been shown

for melanomas and glioblastomas as well as cancers of breast, stomach, pancreas and cervix (14-19).

A functional impact of ß3 integrins on tumor progression and/or invasiveness might explain the observation that their expression positively correlates with a higher risk of patient death. Such a clinical relationship has been prospectively described in 111 patients with melanomas of intermediate thickness (20). Furthermore, the expression of ß3-integrin had a significant prognostic impact on outcome in 82 advanced patients with cervical tumors according to a recent analysis from our group (21).

The molecular mechanisms how ß3 integrins accelerate tumor progression and/or invasiveness, respectively, and thus influence patient survival are only partially understood. The higher concentration of ß3 integrins as functional adhesion receptors on the cell surface might change the binding affinity and specificity of tumor cells to enhance tissue targeting (22). Increased expression of αvß3 integrin in vascular 'hot spots' might also lead to induction of angiogenesis that ensures a better nutrition supply of the transformed tissue (23,24). Furthermore, results from *in vitro* experiments suggest that the interaction of αv ß3 integrin with VEGF (25), MMP-2 (26,27), osteopontin (28) and/or the adhesion molecule CEA-1 (29) might contribute to a ß3 integrin-mediated impact on tumor biology.

The aim of the present study was to ascertain whether the invasive potential of human MDA-MB-231 breast cancer cells depends on the expression of the integrin ß3 subunit. Using the developing chicken CAM as experimental model for cell grafting, we found an increased rate of CAM invasion and a significant MMP-2 up-regulation in these cells compared to mock-transfected cells suggesting that the presence of ß3 integrins in epithelial cells enhances the metastatic potential *in vivo*.

Materials and methods

Cell lines and tumor cell spheroids. Tumor spheroids were prepared from human breast cancer cells (MDA-MB-231), which were either mock-transfected (MDA-MB-231/clone 1.1) or transfected with DNA encoding the gene for human ß3 integrin subunit (MDA-MB-231/ß3 clone 36.4) as described (30). Both cell lines were grown in DMEM containing 10% fetal calf serum and maintained in a humidified 5% CO_2 atmosphere at 37˚C.

For determination of growth rates, 200,000 cells of cell lines 1.1 and 36.4 were plated on plastic petri dishes of 3.5 cm in diameter. On each of the following six days, the cells in three plates were counted after trypsination.

Immunoblotting. The 1.1 and 36.4 cells were solubilised in lysis buffer [1% (v/v) Triton X-100 in PBS, pH 7.4] for 1 h at 4˚C. By centrifugation at 20,000 g for 15 min, insoluble material was removed. Equal amounts of protein of both cell lines were subjected to SDS-PAGE on a 7.5% gel and transferred to nitrocellulose membrane filters. After blocking with 5% (w/v) dry milk powder in washing buffer (0.1% v/v Tween-20 in PBS, pH 7.4), the blot membranes were incubated with a MAb against integrin ß3 subunit (MAB-2008; Chemicon, Hofheim, Germany) diluted 1:500 in washing buffer at 4˚C overnight. The blots were subsequently probed for 1 h with a 1:5,000 dilution of horseradish peroxidase-conjugated secondary antibody from Sigma (Buchs, Switzerland). Unbound primary and secondary antibodies were removed by four incubations (5 min each) in washing buffer. Immunoblots were developed by chemiluminescence as described (31) using an ECL detection kit (Perbio Pierce, Lausanne, Switzerland).

In vitro matrigel degradation assay. 96-well-microtiter plates (BD Falcon, Basel, Switzerland) were blocked with 100 µl of 1% (w/v) BSA in PBS-Tween for 30 min at 37˚C and then washed three times in PBS, pH 7.4. In each well, 10 µl of matrigel (BD Pharmingen, Basel, Switzerland) diluted in 1:3 RPMI medium were adsorbed for 30 min at 37˚C. Subsequently, 20,000 cells in 100 µl of RPMI were cultured at 37˚C for periods as listed in Results. In control experiments, the medium was supplemented with RAD or RGD-peptides (Bachem, Bubendorf, Switzerland) in a final concentration of 100 µM.

For harvesting of cells and matrigel, RPMI was decanted. The wells were washed once with PBS, pH 7.4 and then incubated with 20 µl of solubilisation buffer [1% (w/v) Triton X-100 in PBS, pH 7.4] at 37˚C for 5 min. The supernatants containing the cellular proteins were collected and subjected to immunoblotting with anti-matrix metalloproteinase antibodies (MMP)-2 (Ab-4, NeoMarkers, Fremont, CA, USA) under non-reducing conditions or zymogram analysis after determination of protein concentration. The microtiter plates were transferred on ice, and wells overplayed with 100 µl of BCA reagent (Perbio Pierce) for 30 min and then transferred to 57˚C for protein determination according to the protocol supplied by the manufacturer.

Zymography. Gelatin zymography for the detection of MMP activity was performed as described (32). Equal amounts of proteins (25 µg) from homogenates of 1.1 and 36.4 cells were mixed with non-reducing SDS-PAGE sample buffer and separated on 10% zymogram polyacrylamide gels (Bio-Rad, Reinach, Switzerland) at 4˚C and 100 V. The gels were washed with 0.5% Triton X-100 (v/v) in TBS, pH 7.4 for 15 min, rinsed briefly with TBS, pH 7.4, incubated in TBS, pH 7.4 supplemented with 1 mM Ca^{2+} at 37˚C for 16 h. Staining was performed with Coomassie Brillant Blue and destaining in aqua. dest. Negative staining of transparent bands revealed MMP activity.

Generation of spheroids and their grafting onto chicken CAMs. The shell-free culture method for chicken embryos was used to obtain the chorioallantoic membrane (CAM) as recipient for the grafting experiments (33). After three days of incubation, eggs from Brown Leghorn hens were opened and carefully transferred into plastic petri dishes of 8 cm in diameter. The embryos were incubated at 37˚C in humid atmosphere until grafting of spheroids.

Spheroids as three-dimensional cell aggregates described by Korff and Augustin (34) were generated from both 1.1 and 36.4 cell lines. The 0.12% (w/v) methocel stock solution was prepared from methylcellulose (Sigma) as recommended

at http://www.spherogenex.de/faqculture.htm. Per spheroid, 10,000 trypsinated cells were mixed with 32 μl of DMEM containing 10% (v/v) fetal calf serum and 8 μl of methocel stock solution. The cell suspension was pipetted as drops onto the cover of a petri dish in which about 50 drops could be placed. The dishes were then incubated at 37˚C in humid 5% CO_2 atmosphere. In general, the cells clustered to spheroids within a day. After two days of incubation, the spheroids were carefully grafted onto the surface of E10 day-old CAMs. Regularly, one 1.1 and one 36.4 spheroid were placed simultaneously on each of the CAMs, which were then incubated at 37˚C in humid atmosphere.

Qualitative and quantitative characterisation of spheroid growth on chicken CAMs. For determination of spreading on the CAM surface, the areas where the spheroids had been grafted were photographed with a digital camera (Nikon Coolpix 995) on each of the five days of CAM incubation. The areas occupied by the spheroids were subsequently measured on prints applying the software Analysis (Soft-Imaging Systems, Münster, Germany) and plotted against the time period of incubation.

The invasive potential was defined as the ability of spheroids to penetrate the chorioepithelial layer. To determine the invasion the area inside the CAMs occupied by tumor cells was measured on semi-thin sections of spheroids collected after day 3 and day 5 of incubation using the software Analysis (Soft-Imaging Systems). Since we performed this analysis on serial sections, which were separated 200 μm from each other and encompassed the entire spheroid expansion, we could extrapolate the volume of the spheroids *in toto* as well as inside the CAM. This was done by multiplication of the mean area (mm^2) with the number of serial sections and 0.2 (mm).

For preparation of semi-thin sections, CAM areas (~1 cm^3 in size) with spheroids were excised. These samples were stored in Karnowsky solution (2.5% (v/v) glutaraldehyde, 2% (v/v) paraformaldehyde in 0.1 M sodium cacodylate, pH 7.4) at 4˚C for several days. The samples were extensively washed in cacodylate buffer (0.1 M sodium cacodylate adjusted with HCl to pH 7.4) and post-fixed in 1% (w/v) osmium tetroxide (buffered with 0.1 M sodium cacodylate adjusted to 370 mOsm and pH 7.4) for 2 h. Subsequently, they were washed in maleate buffer (0.05 M maleic acid adjusted with NaOH to pH 5.0), block-stained with uranyl acetate (0.5% w/v in maleate buffer), dehydrated in ethanol and embedded in Epon 812 (Fluka, Buchs, Switzerland) as reported previously (33). Sections (1 μm) were prepared by using histo-diamant knives, stained with Toluidine Blue and analyzed using a Leica Leitz DMR light microscope.

Immunohistochemistry. The samples were fixed in 4% (v/v) paraformaldehyde and embedded in paraffin as described previously (33). Using a HM 355S microtom (Microm, Walldorf, Germany), 3-μm thick paraffin sections were cut and transferred to gelatinized slides which were air-dried at 60˚C overnight. Subsequently, they were dewaxed in xylene, rehydrated in ethanol and rinsed twice in TBS (Tris-buffered saline adjusted with HCl to pH 7.4). Prior to incubation with antibodies, the sections were bathed in 0.01 M sodium citrate (pH 6.0) and heated in a microwave oven (180 W) for 15 min.

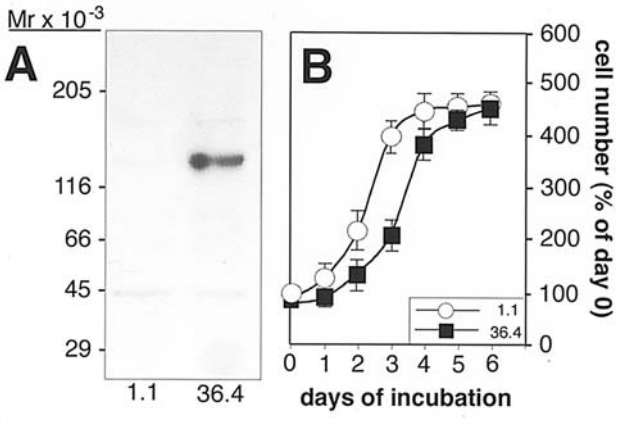

Figure 1. 36.4 cells express constitutively ß3 integrin subunit and grow with similar rates as 1.1 cells A, Solubilisates were prepared from cultured cells, which were either transfected with human ß3 integrin subunit (36.4) or mock-transfected (1.1). After SDS-PAGE under non-reducing conditions, the immunoblot matrices were probed with an antibody against ß3 integrin subunit (MAb 2008, Chemicon). B, Growth rates of 1.1 and 36.4 cells were determined during six days period of culturing. These results indicate that the cell line 36.4 transfected with the gene for ß3 integrin subunit but not the mock-transfected cell line 1.1 expresses constitutively the ß3 integrin subunit. Furthermore, comparison of the growth rates of cell lines 1.1 and 36.4 during six days of incubation revealed that both cell lines have similar proliferation activities.

After blocking with 1% (w/v) casein in TBS for 10 min, sections were incubated with the monoclonal antibody (MAb) against MMP-2 (Ab-4, NeoMarkers) at 4˚C overnight which was applied in a ready-to-use dilution.

After intensive washing, the sections were exposed to an affinity-purified biotinylated secondary antibody (Dako, Glostrup, Denmark) for 45 min at RT, which was diluted 1:200 in TBS, subsequently washed three times in TBS and then incubated with an avidin-biotin horseradish peroxidase-complex (Dako) for 45 min at RT which was diluted 1:500 in TBS. For visualization of the reaction product, sections were exposed to 0.02 (w/v) 3-amino-9-ethylcarbazole (Sigma) for 1-12 min. Extensive washing in aqua dest. stopped the histochemical reaction. The sections were then counter-stained with hematoxylin and cover-slipped in Aquatex (Merck, Darmstadt, Germany).

Statistical analyses. Data were expressed as means ± standard deviation. For comparison of the mean values, the paired Student's t-test was used. A significance level of $p \leq 0.05$ was assumed for all statistical evaluations.

Results

Performing immunoblotting with a MAb against anti-ß3 integrin subunit, detergent extracts of 36.4 cells exhibited a strong 130-kDa band whereas no immunoreactive protein was detected in 1.1 cell extracts after SDS-PAGE under non-reducing conditions (Fig. 1A). These results indicate that the cell line 36.4 transfected with the gene for ß3 integrin subunit but not the mock-transfected cell line 1.1 expresses constitutively the ß3 integrin subunit. Furthermore, comparison of the growth rates of cell lines 1.1 and 36.4 during six days

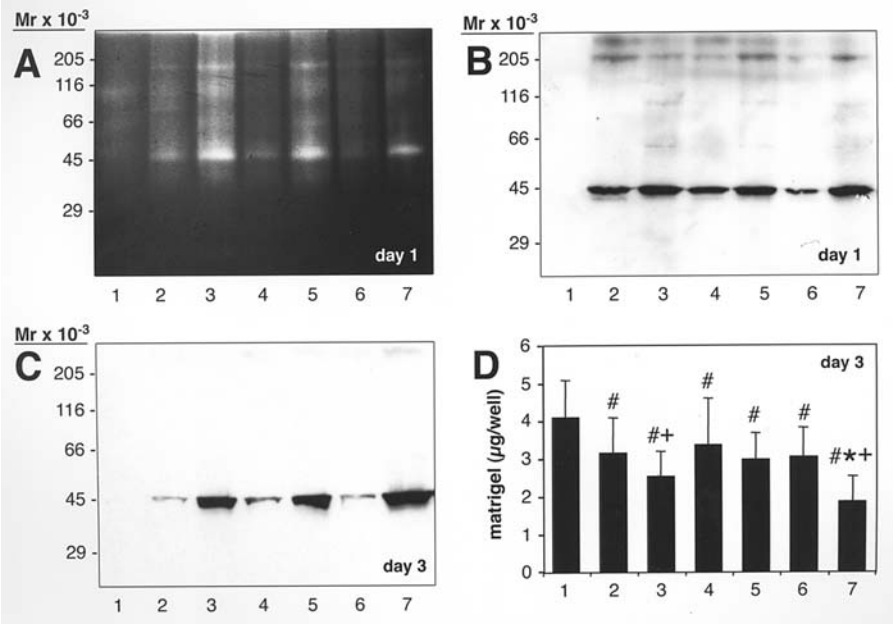

Figure 2. Matrigel degradation activity and MMP-2 expression are higher in integrin ß3-subunit 36.4 cells than mock-transfected 1.1 cells. 20,000 cells of cell line 1.1 or 36.4 were cultured for 1 day (A and B), 3 days (C and D) on matrigel. After incubated, wells were first extracted with 1% Triton X-100 at 37˚C for solubilisation of cells. A second extraction step at 4˚C was performed to obtain the undigested matrigel remaining adsorbed in the wells of the microtiter plates. A, Gelatin zymography (10% gel) in the presence of 1 mM Ca^{2+} for demonstration of MMP activity. B and C, Immunoblot (12.5% gel) with anti-MMP-2 antibodies on cell extracts incubated for 1 day (B) and 3 days (C) on matrigel. Note the presence of high molecular weight bands in B presumably non-specifically developed but indicating similar loading of the lanes. D, After 3 days of culturing on identical amounts of matrigel, 1.1 and 36.4 cells were removed. Remaining matrigel was collected and quantified to determine the cell-associated degradation activity during incubation. 1, matrigel; 2, matrigel with 1.1 cells; 3, matrigel with 36.4 cells; 4, matrigel with 1.1 cells and 100 μM RAD-peptid; 5, matrigel with 36.4 cells and 100 μM RAD-peptide; 6, matrigel with 1.1 cells and 100 μM RGD-peptide; and 7, matrigel with 36.4 cells and 100 μM RGD-peptide. $^{\#}$Values significantly different from matrigel without cells, *Values from 36.4 cells significantly different from the values for 1.1 spheroids incubated under the same conditions; and $^{+}$values from cells cultured in the presence of a peptide significantly different from the values for the same cell incubated without peptide.

of incubation revealed that both cell lines have similar proliferation activities (Fig. 1B).

For determination of matrix metalloproteinase (MMP) activity in 1.1 and 36.4 cells, gelatin zymography was performed using extracts from both cell lines, which had been cultured on matrigel for one day. Comparison of the band patterns observed in the zymograms (Fig. 2A) showed that extracts of 36.4 cells contained a strong MMP activity at 45 kDa and a weaker one at 180 kDa. These bands were also detected in extracts of 36.4 cells, which were incubated in the presence of the integrin substrate peptide RGD or the control peptide RAD. We subjected the same extracts to immunblotting with anti-MMP2 antibodies finding an immunoreactive band at 45 kDa in all samples which appeared more distinctive in samples from 36.4 than 1.1 cells (Fig. 2B). After 3 days of incubation (Fig. 2C), 36.4 cell extracts clearly exhibited stronger MMP-2 immunoreactivity than 1.1 cell extracts. Furthermore, the expression of MMP-2 was higher when 36.4 cells were incubated with the RGD peptide as in 36.4 cells incubated without peptide or with RAD peptide. Analysing the amount of matrigel remaining in the wells of the microtiter plate, matrigel was significantly lower in those wells in which 36.4 cells had been cultured compared to those in which 1.1 cells were grown (Fig. 2D). The matrigel amount determined was lowest in the wells of 36.4 cells incubated in the presence of the RGD peptide. In summary, the data from the *in vitro* matrigel degradation assay show that 36.4 cells express higher concentrations of MMP-2 which is apparently further up-regulated in the presence of RGD-peptide.

For detection of *in vivo* effects of constitutive expression of ß3 integrin subunit spheroids of both cell lines were grafted on the surface of 10 day-old chicken CAMs. The areas of the CAMs where the spheroids had been placed were photographed on each of the five following days of incubation. Both 1.1 and 36.4 spheroids showed a similar pattern of spreading on the CAM surface (Fig. 3A). At day 1 of incubation, all spheroids remained small and were barely detectable but expanded massively at day 2, then covering large areas of the CAM surface. In many cases, the spheroid spreading followed the course of a larger vessel in the depth of the CAM. Between days 3 and 5 of incubation, the CAM surface covered by the spheroids was not further enlarged. We rather observed a condensation of the spheroids, which was characterised by a slight decrease in CAM area coverage and an apparently denser core. Furthermore, some spheroids of both cell lines exhibited an angio-attractive effect since larger vessels apparently changed their direction toward the spheroid losing their tortuous appearance.

We quantified the sizes of the CAM surface being covered by the spheroids during incubation (Fig. 3B). Both 1.1 and 36.4 spheroids expanded rapidly after day 1 of incubation. Reaching a maximum between day 2-3 of incubation, the mean CAM area occupied by 1.1 spheroids declined con-

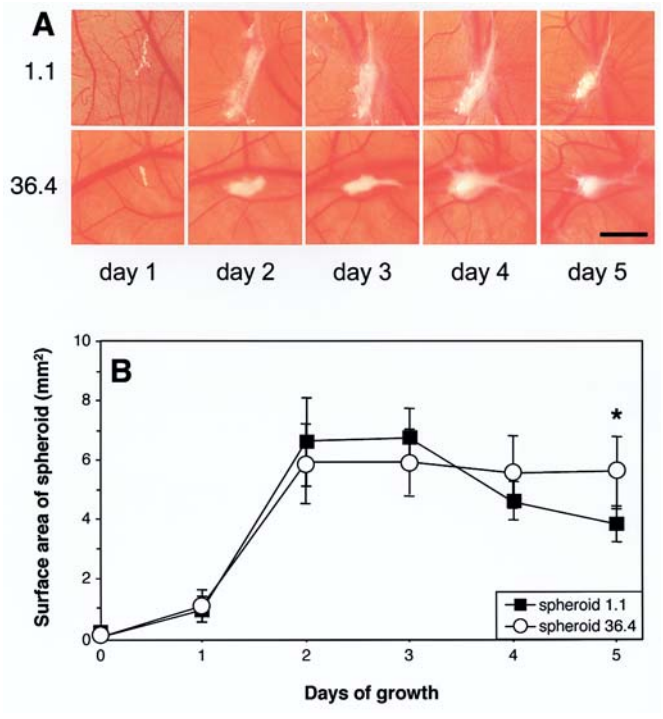

Figure 3. Spreading of spheroids on the surface of chicken CAM analysed during the five-day period of incubation. A, Spheroids consisting of 10,000 cells each from cell lines 1.1 and 36.4 were grafted onto E10 chicken CAM. On each day of the incubation, the growth of the spheroids on the CAM surface was monitored with a digital camera. Bar in the lower right photo-imagine (day 5; spheroid 36.4) represents 4 mm. B, The sizes of the CAM surface coverage of the spheroids were quantified and plotted against the day of incubation. n=6 for each cell line. Values represent means ± standard deviation. *Values from 36.4 spheroids significantly different from the values for 1.1 spheroids obtained at the same day of incubation.

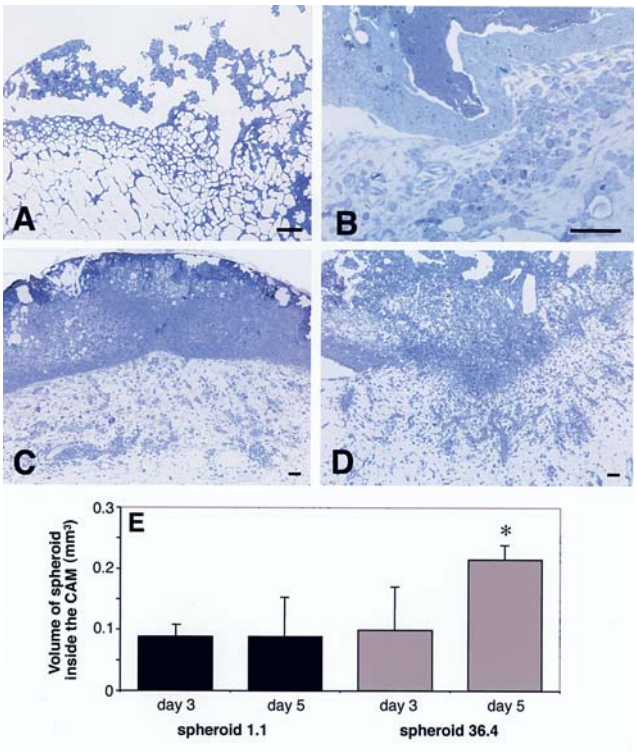

Figure 4. Invasion of spheroids into chicken CAM during five days of incubation. Spheroids from both cell lines consisting of 10,000 cells were placed on E10 chicken CAM and harvested after either 3 days or 5 days of incubation. Semi-thin sections were prepared from these spheroids and stained with Toluidine Blue. A, Overview of a 2d-old 36.4 spheroid on the CAM; B, Detail of a 1.1 spheroid after day 3 of incubation showing extensive proliferation of the chorioepithelial layer and extensive mesenchymal dis-organisations; C, Overview of a 5d-old 1.1 spheroid unable to penetrate the thickened chorioepithelial layer; and D, Overview of a 36.4 spheroid after 5 days of incubation invading the CAM. Bars represent 50 μm. E, The volumes of 1.1 and 36.4 spheroids inside the CAMs were plotted against the duration of incubation. Volumes were calculated from cross-sections of CAMs obtained ay 200 μm distance on which the sizes of the areas occupied by spheroid cells inside the CAM were determined. n=4 for each cell line and time-point. Values represent means ± standard deviation. *Values significantly different from the value for 1.1 spheroids obtained at day 3 of incubation.

tinuously within the next two days whereas the mean covered area of 36.4 spheroids changed only slightly during this period. After day 5 of incubation 1.1 spheroids covered a CAM area of 3.9±0.6 mm^2 on average while the mean area covered by 36.4 spheroids (5.6±1.2 mm^2) was significantly larger ($p \leq 0.05$).

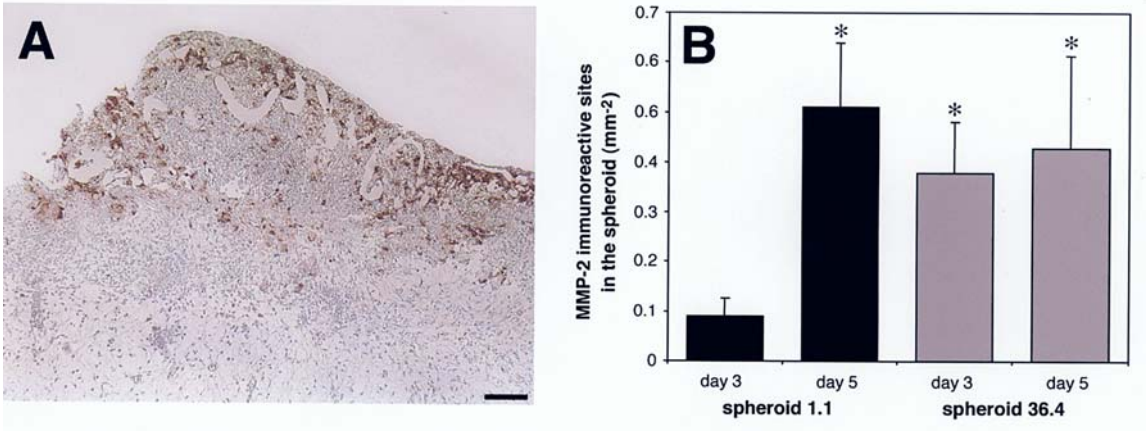

Figure 5. Expression of MMP-2 in 1.1 and 36.4 spheroids collected on the third and fifth day of incubation after grafting onto chicken CAM. A, Paraffin-embedded sections were incubated with anti-MMP-2 antibodies showing strong and exclusive immunoreactivity in individual spheroid cells outside the chorioepithelial layer as exemplified for a 36.4 spheroid collected after day 5 of incubation. B, The numbers of MMP-2-immunoreactive sites were counted and set in relation to the sizes of the spheroids detected on the sections. n= at least five sections from each of three spheroids for every time-point and cell type. Values represent means ± standard deviation. *Values significantly different from the value for 1.1 spheroids obtained at day 3 of incubation. Bar represent 50 μm.

Toluidine Blue-stained semi-thin sections were used to characterise the spheroid growth into the CAMs (Fig. 4). At day 2 of incubation, all spheroids were detected as thin cell layer (~60 μm thick) on the top of the CAM which was enlarged due to edema (Fig. 4A). At day 3 of incubation, CAMs reacted with extensive proliferation of the chorio-epithelial layer and large mesenchymal dis-organisations against the compact spheroid aggregates on the surface (Fig. 4B). At day 5 of incubation, 1.1 spheroids were mainly not able to penetrate the thickened chorioepithelial layer (Fig. 4C), whereas 36.4 spheroids were detected inside the CAM at many sites (Fig. 4D).

We also determined the invasive potential of 1.1 and 36.4 spheroids by quantification of the spheroid proportion inside the CAM. As shown in Fig. 4E, the mean volume of 1.1 spheroids detected inside the CAM remained almost unchanged at days 3 and 5 of incubation (0.09±0.03 mm^3 versus 0.08±0.07 mm^3). In contrast, 36.4 spheroids occupied a mean volume of 0.08±0.10 mm^3 at day 3 of incubation which was significantly enlarged to 0.19±0.05 mm^3 at day 5 of incubation ($p \leq 0.05$). The volumes of the spheroids *in toto* were 0.37±0.18 mm^3 (1.1; day 3), 0.15±0.13 mm^3 (1.1; day 5), 0.38±0.11 mm^3 (36.4; day 3) and 0.42±0.14 mm^3 (36.4; day 5). Correspondingly, the proportion of spheroids inside the CAM was ~24 % (1.1; day 3), 53% (1.1; day 5), 29% (36.4; day 3) and 45% (36.4; day 5). Thus, the invasive potential increased for both spheroid types from days 3 to 5 of incubation. Since, however, the 36.4 spheroids apparently grew much faster than the spheroids consisting of 1.1 cells, ß3 integrin expressing cells had a higher capability than mock-transfected control spheroid tumor cells to enter the CAM.

An immunohistochemical analysis with anti-MMP-2 antibodies was performed on 1.1 and 36.4 spheroids grafted onto the CAM surface, which were collected after days 3 and 5 of incubation. As shown in Fig. 5A, MMP-2 immunoreactive sites were restricted to individual spheroid cells outside the chorioepithelial layer while no MMP-2 immunoreactivity was detected in the CAM. Semi-quantitative analysis counting the numbers of MMP-2 immunoreactive sites in the area occupied by the spheroids revealed that significantly less sites in 1.1 spheroids expressed MMP-2 at day 3 (0.09±0.01 mm^{-2}) than at day 5 (0.61±0.13 mm^{-2}) of incubation (Fig. 5B). In contrast, 36.4 spheroids contained significant more MMP-2 immunoreactive sites at day 3 of incubation (0.38±0.15 mm^{-2}) than 1.1 spheroids. The concentration of MMP-2 immuno-reactive sites in 36.4 spheroids was only slightly increased at day 5 of incubation (0.43±0.25 mm^{-2}).

Discussion

Our *in vivo* assay revealed that the spheroids of MDA-MB-231 breast cancer cells that were transfected with the ß3 integrin subunit (36.4 cells) expanded after 5 days of incubation significantly more on the surface of the CAM than the spheroids consisting of mock transfected cells (1.1 cells). Furthermore, only 36.4 but not 1.1 cells were detected inside the CAM suggesting that the presence of ß3 enabled the tumor cells to invade the epithelial barrier. We conclude that the presence of the ß3 integrin in MDA-MB-231 breast cancer cells leads to higher incidence of proliferation and allows invasion of the CAM.

The conclusion that the ß3 integrins increase the metastatic potential of carcinoma cells is supported by the observation that disaggregated cells from freshly dissected breast cancer metastases contained high levels of αv ß3 as well as c-erbB-2 receptor, MMP-2, CD44 and α6 integrin (35). Furthermore, a substantial *in vivo* affect of ß1 and ß3-integrins on the regulation of survival, adhesion, invasion and MMP-2 activity has been found in four glioblastoma cell lines using anti-integrin antibodies, disintegrin echistatin and MMP inhibitors (36). However, ß3/ß5 integrin-independent mechanisms might also contribute to the metastatic potential, as no major differences were observed in size or number of lung metastases in ß integrin deficient-mice compared to control mice (37).

Our *in vitro* assay combines the quantitative recording of gelatin zymography, anti-MMP-2-immunoblotting as well as matrigel degradation activity. We found significantly more MMP-2 expression and activity in 36.4 cells than in 1.1 cells indicating that the presence of the ß3 integrin subunit triggers the increased induction and secretion of active MMP-2 in MDA-MB-231 breast cancer cells. Correspondingly, a significantly lower number of immunohistochemically MMP-2-reactive sites were detected in 1.1 spheroids compared to 36.4 spheroids.

Other studies support the conclusion that MMP-2 is up-regulated in response to ß3 integrin expression. The αvß3 integrin is bound to MMP-2 in a functionally active form on the cell surface of angiogenic blood vessels and melanoma tumors (38). A ß integrin/MMP-2 interaction could be shown for tumor cells, e.g. cervical (27) and ovarian cancer cells (39). Assembled as complex, conformational changes of αvß3 initiated by extracellular ligand binding are transduced intracellularly to induce a higher transcription rate of MMP-2 and the potentiation of its matrix degradation activity to promote invasive activity (38). Such a ß3 integrin subunit-mediated outside-in signaling function has been previously assigned to an 11 amino acid long stretch within the cytoplasmic domain (40).

We observed a stronger up-regulation of MMP-2 activity when the 36.4 cells were incubated in the presence of the RGD-peptide suggesting that this molecule that mimics an integrin ligand enhances integrin signaling activity resulting in MMP-2 induction. A similar observation was made before (41) describing that the GRGDSP peptide induced the secretion of higher MMP-2 levels in human GCT23 giant cell tumor cells. However, incubation of metastatic 4T1 cells with the RGD-peptide decreased MMP-2 expression (42). No effect of RGD-peptides on MMP-2 expression but up-regulation of MMP-1 and MMP-3 was found in cultured human dermal fibroblasts (43). Thus, it apparently depends on the cell type or the experimental microenvironment whether RGD-based reagents might inhibit or activate integrin mediated outside-in signaling (in our case MMP-2 expression). This conclusion is in accordance with recent reflections hypothesizing that reagents (antibodies, small molecules) that block integrin interactions with a solid-phase substrate could nonetheless be agonists of signal transduction by the same integrins (44).

Our experimental approach, applying tumor cell spheroids on the developing CAM, represents an easy and clear results

delivering system to analyse the invasion potential *in vivo*. This approach might be used for further studies, e.g. to determine optimal targets for therapeutic strategies, especially the role of integrin blockage.

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References

1. Hynes RO: Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69: 11-25, 1992.
2. Arnaout MA, Mahalingam B and Xiong JP: Integrin structure, allostery, and bidirectional signaling. Annu Rev Cell Dev Biol 21: 381-410, 2005.
3. Miyamoto S, Teramoto H, Coso OA, Gutkind JS, Burbelo PD, Akiyama SK and Yamada KM: Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. J Cell Biol 131: 791-805, 1995.
4. Van der Flier A and Sonnenberg A: Function and interactions of integrins. Cell Tissue Res 305: 285-298, 2001.
5. Parise LV, Lee J and Juliano RL: New aspects of integrin signaling in cancer. Semin Cancer Biol 10: 407-414, 2000.
6. Kinbara K, Goldfinger LE, Hansen M, Chou FL and Ginsberg MH: Ras GTPases: integrins' friends or foes? Nat Rev Mol Cell Biol 4: 767-776, 2003.
7. Shattil SJ: Function and regulation of the beta 3 integrins in hemostasis and vascular biology. Thromb Haemost 74: 149-155, 1995.
8. Chen YQ, Trikha M, Gao X, Bazaz R, Porter AT, Timar J and Honn KV: Ectopic expression of platelet integrin alphaIIb beta3 in tumor cells from various species and histological origin. Int J Cancer 72: 642-648, 1997.
9. Carreiras F, Denoux Y, Staedel C, Lehmann M, Sichel F and Gauduchon P: Expression and localization of alpha v integrins and their ligand vitronectin in normal ovarian epithelium and in ovarian carcinoma. Gynecol Oncol 62: 260-267, 1996.
10. Van Belle PA, Elenitsas R, Satyamoorthy K, Wolfe JT, Guerry DT, Schuchter L, van Belle TJ, Albelda S, Tahin P, Herlyn M and Elder DE: Progression-related expression of beta3 integrin in melanomas and nevi. Hum Pathol 30: 562-567, 1999.
11. Switala-Jelen K, Dabrowska K, Opolski A, Lipinska L, Nowaczyk M and Gorski A: The biological functions of beta3 integrins. Folia Biol (Praha) 50: 143-152, 2004.
12. Trikha M, Timar J, Zacharek A, Nemeth JA, Cai Y, Dome B, Somlai B, Raso E, Ladanyi A and Honn KV: Role for beta3 integrins in human melanoma growth and survival. Int J Cancer 101: 156-167, 2002.
13. Brakebusch C, Bouvard D, Stanchi F, Sakai T and Fassler R: Integrins in invasive growth. J Clin Invest 109: 999-1006, 2002.
14. Albelda SM, Mette SA, Elder DE, Stewart R, Damjanovich L, Herlyn M and Buck CA: Integrin distribution in malignant melanoma: association of the beta 3 subunit with tumor progression. Cancer Res 50: 6757-6764, 1990.
15. Chattopadhyay N and Chatterjee A: Studies on the expression of alpha(v)beta3 integrin receptors in non-malignant and malignant human cervical tumor tissues. J Exp Clin Cancer Res 20: 269-275, 2001.
16. Gingras MC, Roussel E, Bruner JM, Branch CD and Moser RP: Comparison of cell adhesion molecule expression between glioblastoma multiforme and autologous normal brain tissue. J Neuroimmunol 57: 143-153, 1995.
17. Hosotani R, Kawaguchi M, Masui T, Koshiba T, Ida J, Fujimoto K, Wada M, Doi R and Imamura M: Expression of integrin alphaVbeta3 in pancreatic carcinoma: relation to MMP-2 activation and lymph node metastasis. Pancreas 25: E30-E35, 2002.
18. Kawahara E, Ooi A and Nakanishi I: Integrin distribution in gastric carcinoma: association of beta 3 and beta 5 integrins with tumor invasiveness. Pathol Int 45: 493-500, 1995.
19. Pignatelli M, Cardillo MR, Hanby A and Stamp GW: Integrins and their accessory adhesion molecules in mammary carcinomas: loss of polarization in poorly differentiated tumors. Hum Pathol 23: 1159-1166, 1992.
20. Hieken TJ, Ronan SG, Farolan M, Shilkaitis AL and Das Gupta TK: Molecular prognostic markers in intermediate-thickness cutaneous malignant melanoma. Cancer 85: 375-382, 1999.
21. Gruber G, Hess J, Stiefel C, Aebersold DM, Zimmer Y, Greiner RH, Studer U, Altermatt HJ, Hlushchuk R and Djonov V: Correlation between the tumoral expression of beta3-integrin and outcome in cervical cancer patients who had undergone radiotherapy. Br J Cancer 92: 41-46, 2005.
22. Ria R, Vacca A, Ribatti D, Di Raimondo F, Merchionne F and Dammacco F: Alpha(v)beta(3) integrin engagement enhances cell invasiveness in human multiple myeloma. Haematologica 87: 836-845, 2002.
23. Gasparini G, Brooks PC, Biganzoli E, Vermeulen PB, Bonoldi E, Dirix LY, Ranieri G, Miceli R and Cheresh DA: Vascular integrin alpha(v)beta3: a new prognostic indicator in breast cancer. Clin Cancer Res 4: 2625-2634, 1998.
24. Vonlaufen A, Wiedle G, Borisch B, Birrer S, Luder P and Imhof BA: Integrin alpha(v)beta(3) expression in colon carcinoma correlates with survival. Mod Pathol 14: 1126-1132, 2001.
25. De S, Razorenova O, McCabe NP, O'Toole T, Qin J and Byzova TV: VEGF-integrin interplay controls tumor growth and vascularization. Proc Natl Acad Sci USA 102: 7589-7594, 2005.
26. Hofmann UB, Westphal JR, Waas ET, Becker JC, Ruiter DJ and van Muijen GN: Coexpression of integrin alpha(v)beta3 and matrix metalloproteinase-2 (MMP-2) coincides with MMP-2 activation: correlation with melanoma progression. J Invest Dermatol 115: 625-632, 2000.
27. Chatterjee N and Chatterjee A: Role of alphavbeta3 integrin receptor in the invasive potential of human cervical cancer (SiHa) cells. J Environ Pathol Toxicol Oncol 20: 211-221, 2001.
28. Furger KA, Allan AL, Wilson SM, Hota C, Vantyghem SA, Postenka CO, Al-Katib W, Chambers AF and Tuck AB: Beta(3) integrin expression increases breast carcinoma cell responsiveness to the malignancy-enhancing effects of osteopontin. Mol Cancer Res 1: 810-819, 2003.
29. Brummer J, Ebrahimnejad A, Flayeh R, Schumacher U, Loning T, Bamberger AM and Wagener C: cis interaction of the cell adhesion molecule CEACAM1 with integrin beta(3). Am J Pathol 159: 537-546, 2001.
30. Pecheur I, Peyruchaud O, Serre CM, Guglielmi J, Voland C, Bourre F, Margue C, Cohen-Solal M, Buffet A, Kieffer N and Clezardin P: Integrin alpha(v)beta3 expression confers on tumor cells a greater propensity to metastasize to bone. FASEB J 16: 1266-1268, 2002.
31. Baum O, Da Silva-Azevedo L, Willerding G, Wockel A, Planitzer G, Gossrau R, Pries AR and Zakrzewicz A: Endothelial NOS is main mediator for shear stress-dependent angiogenesis in skeletal muscle after prazosin administration. Am J Physiol Heart Circ Physiol 287: H2300-H2308, 2004.
32. Bermpohl F, Loster K, Reutter W and Baum O: Rat dipeptidyl peptidase IV (DPP IV) exhibits endopeptidase activity with specificity for denatured fibrillar collagens. FEBS Lett 428: 152-156, 1998.
33. Djonov V, Schmid M, Tschanz SA and Burri PH: Intussusceptive angiogenesis: its role in embryonic vascular network formation. Circ Res 86: 286-292, 2000.
34. Korff T and Augustin HG: Integration of endothelial cells in multicellular spheroids prevents apoptosis and induces differentiation. J Cell Biol 143: 1341-1352, 1998.
35. Roetger A, Merschjann A, Dittmar T, Jackisch C, Barnekow A and Brandt B: Selection of potentially metastatic subpopulations expressing c-erbB-2 from breast cancer tissue by use of an extravasation model. Am J Pathol 153: 1797-1806, 1998.
36. Cordes N, Hansmeier B, Beinke C, Meineke V and van Beuningen D: Irradiation differentially affects substratum-dependent survival, adhesion, and invasion of glioblastoma cell lines. Br J Cancer 89: 2122-2132, 2003.
37. Taverna D, Crowley D, Connolly M, Bronson RT and Hynes RO: A direct test of potential roles for beta3 and beta5 integrins in growth and metastasis of murine mammary carcinomas. Cancer Res 65: 10324-10329, 2005.

38. Brooks PC, Stromblad S, Sanders LC, von Schalscha TL, Aimes RT, Stetler-Stevenson WG, Quigley JP and Cheresh DA: Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. Cell 85: 683-693, 1996.
39. Leroy-Dudal J, Demeilliers C, Gallet O, Pauthe E, Dutoit S, Agniel R, Gauduchon P and Carreiras F: Transmigration of human ovarian adenocarcinoma cells through endothelial extracellular matrix involves alphav integrins and the participation of MMP2. Int J Cancer 114: 531-543, 2005.
40. Morgan MR, Thomas GJ, Russell A, Hart IR and Marshall JF: The integrin cytoplasmic-tail motif EKQKVDLSTDC is sufficient to promote tumor cell invasion mediated by matrix metalloproteinase (MMP)-2 or MMP-9. J Biol Chem 279: 26533-26539, 2004.
41. Teti A, Farina AR, Villanova I, Tiberio A, Tacconelli A, Sciortino G, Chambers AF, Gulino A and Mackay AR: Activation of MMP-2 by human GCT23 giant cell tumour cells induced by osteopontin, bone sialoprotein and GRGDSP peptides is RGD and cell shape change dependent. Int J Cancer 77: 82-93, 1998.
42. Mi Z, Guo H, Wai PY, Gao C and Kuo PC: Integrin-linked kinase regulates osteopontin-dependent MMP-2 and uPA expression to convey metastatic function in murine mammary epithelial cancer cells. Carcinogenesis 27: 1134-1145, 2006.
43. Booms P, Pregla R, Ney A, Barthel F, Reinhardt DP, Pletschacher A, Mundlos S and Robinson PN: RGD-containing fibrillin-1 fragments upregulate matrix metalloproteinase expression in cell culture: a potential factor in the pathogenesis of the Marfan syndrome. Hum Genet 116: 51-61, 2005.
44. Hynes RO: A reevaluation of integrins as regulators of angiogenesis. Nat Med 8: 918-921, 2002.