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DNAzyme as an efficient tool to modulate invasiveness of human carcinoma cells

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In this study we evaluated efficiency of DNAzymes to modulate motility of cancer cells, an important factor in the progression and metastasis of cancers. For this purpose we targeted β1 integrins that are predominant adhesive receptors in various carcinoma cell lines (CX1.1, HT29, LOVO, LS180, PC-3). To evaluate invasiveness of cancer cells, we used a *transwell migration* assay that allowed analyzing chemotactic migration of colon carcinoma cell lines across an ECM-coated membrane. Their adhesive properties were also characterized by the analysis of adhesion to fibronectin, laminin and collagen. In addition, the expression of major integrin subunits, selected intact β1 integrins, and other adhesive receptors (ICAM, E-selectin, uPAR) was analyzed by flow cytometry. Inhibition of β1 integrin expression by DNAzyme to β1 mRNA almost abolished the invasiveness of the CX1.1, HT29, LS180, LOVO and PC-3 cells *in vitro*. These data show that DNAzymes to β1 integrin subunit can be used to inhibit invasiveness of carcinoma cells.

Keywords: DNAzyme, integrin, cancer cells

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INTRODUCTION

Local invasion of the host tissue and metastasis are hallmark features of cancer progression (Hanahan & Weinberg, 2000) and understanding the tumor cell motility is important for therapeutic targeting of cancer progression (Quaranta, 2002). Invasion of tumor cells into the host tissue is regulated by the matrix microenvironment at the tumor-host tissue interface (Liotta & Kohn, 2001; Quaranta, 2002). Migration of cancer cells is likely initiated by cytokines and growth factors released by the host tissue that attract the malignant tumor cells to invade it (Fidler *et al.,* 1998; Kassis *et al.,* 2001; Radisky *et al.,* 2001). Degradation and remodeling of the peri-tumor ECM is considered a necessary step in local tumor invasion (Stetler-Stevenson *et al.,* 1993; Egeblad & Werb, 2002).

In our recent works, we downregulated β1 integrins in several cell lines (Papiewska-Pajak & Antoszczyk, 2006) and provided evidence that DNAzymes can be useful gene-inactivating agents to control expression of membrane proteins (Haier *et al.,* 1998; Sawhney *et al.,* 2002). DNAzymes are commonly used in cancer therapy (Dass *et al.,* 2008; Chan & Khachigian, 2009). DNAzymes to β1 and β3 mRNAs appeared to be strong inhibitors of angiogenesis in *in vitro* assays that might ultimately provide a therapeutic means to inhibit angiogenesis *in vivo*.

Integrin receptors play a primary role in motility of cancer cells. They are involved not only in the physical tethering of the cells to the matrix, but also in transmitting signals in both directions in the cells (outside-in, and inside-out). Changes in expression of integrins induced by carcinogenesis are complex (Goel *et al.,* 2008): (**a**) expression levels of numerous integrins can be upregulated during cancer progression (Hodgkinson *et al.,* 2000; Yuan *et al.,* 2000; Ise *et al.,* 2001; Schaffert *et al.,* 2001); (**b**) in some cells carcinogenesis is associated with a switch in integrin expression, for example, the major integrins α5β1 and α6β1 on normal hepatocytes are replaced by α1β1, α2β1 and α3β1, abundantly expressed in cancer cells (Kawakami-Kimura *et al.,* 1997; Giannelli *et al.,* 2001; Torimura *et al.,* 2001). This switch of integrin expression is associated with the acquisition of migratory capacity, suggesting an important role of these integrins in cancer cell motility. Furthermore, several growth factors that are abundantly expressed in the tumor microenvironment are considered potent stimulators of integrin expression (Kagami *et al.,* 1996; Smida *et al.,* 2000).

The purpose of this study was to test whether DNAzyme to β1 integrin subunit can inhibit pathogenic cell-ECM interactions and thus block invasion of cells in *in vitro* assays.

EXPERIMENTAL PROCEDURES

Materials. Monoclonal antibodies to integrins were from Dako, BD-PharMingen, Serotec Inc., Chemicon International Inc., American Diagnostica Inc.

Synthesis of DNAzyme to β1 mRNA. DNAzyme was chemically synthesized on solid support using an ABI-394 DNA Synthesizer as described before (Cierniewski *et al.,* 1995). This particular DNA sequence (5' CAAGGTGAG$g_1g_2c_3t_4a_5g_6c_7t_8a_9c_{10}a_{11}a_{12}c_{13}g_{14}a_{15}$AATAGAAG 3') was used by us previously to analyze enzymatic activity, specificity, exonuclease resistance, and ability to inhibit expression of β1 integrins in endothelial cells (Cieslak *et al.,* 2002). To increase the stability of the

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Abbreviations: BSA, bovine serum albumin; DNAzyme, DNA-cleaving deoxyribozyme; ECM, extracellular matrix; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; ICAM, intercellular adhesion molecule; PE, phicoerytrin; uPAR, urokinase plasminogen activator receptor.

DNAzyme, oligonucleotides modified with 2'-O-methyl groups were introduced at both the 5' and 3' ends. Thus, the analogue of β1DE called β1DE-OME was used throughout this work. In addition, inactive DNAzyme, β1DE-OME_{IN} with the designed structure based on the inactive antisense oligodeoxynucleotide β1(358–375) (TTCTTTATA $g_1g_2c_3t_4a_5g_6c_7t_8a_9c_{10}a_{11}a_{12}c_{13}g_{14}a_{15}$TCTTTGGAG) was synthesized and used in control experiments. All deoxyribooligonucleotides and DNAzymes were purified by semipreparative RP-HPLC (Hamilton PRP1) (to 98%) and their purity was confirmed by polyacrylamide gel electrophoresis under denaturing conditions.

Carcinoma cell lines and culture conditions. Human colon cancer cell lines (CX1.1, HT29, LOVO, LS180) and human prostate carcinoma cell line (PC-3) were obtained from the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (Wroclaw, Poland). Colon carcinoma cells were cultured and maintained in the MEM-α medium supplemented with 10% fetal bovine serum, streptomycin (100 μg/ml), penicillin (100 units/ml), L-glutamine (2 mM). PC-3 cells were cultured and maintained in DMEM supplemented with 10% fetal bovine serum, streptomycin (100 μg/ml), penicillin (100 units/ml). All cell cultures were maintained at 37°C in the presence of 5% CO_2. For experiments, cells were transferred to 6-well dishes and used at 70% confluency in DMEM or MEM-α supplemented as above. DNAzymes were mixed with LipofectAMINE™ Reagent (Gibco BRL®) suspended in Opti-MEM reduced medium (5 μg/ml) containing 5 mM $MgCl_2$ and diluted with the corresponding medium to obtain the final concentration of 0.5 μM. Transfection was performed according to the manufacturer's protocol. After incubation for 24 h, cells were detached with trypsin/EDTA and used for experiments. Cell viability was determined microscopically by Trypan Blue exclusion and only cell cultures having less than 1% dead cells were included into the study.

Flow cytometry. Cell membrane expression of α4, α5, α6, αv, β1, β3, and β4 integrin subunits, α2β1 and α5β1 as well as uPAR, E-selectin and ICAM was measured by flow cytometry. Subconfluent cells were harvested with trypsin/EDTA and washed with FBS-free MEM-α or DMEM. Then, cells (1×10^6) were suspended in the medium containing 1% bovine serum albumin (BSA) and incubated for 30 min at 4°C in the dark with FITC- or PE-conjugated monoclonal antibodies to the surface receptors. After double washing with 1% BSA/PBS, the cells were fixed by mixing the sedimented cells with 1% paraformaldehyde in PBS and resuspended in FACS Flow liquid. Cell fluorescence was measured with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). The data were analyzed with PC Lysis II software.

Western blotting. Subconfluent cells were grown in the presence or absence of β1DE-oME or β1DE-oME_{IN} for 6 h. After further incubation for 12 h in fresh medium supplemented with 5% FCS the cells were washed with PBS and lysed in M-PER Mammalian Protein Extraction Reagent (Pierce) supplemented with protein inhibitor cocktail (Complete™). Total protein extract (30 μg) from cells transfected with β1DE-OME, β1DE-OME_{IN} or mock-transfected was boiled with 5× concentrated sample buffer with 2-mercaptoethanol and separated by SDS/PAGE and electroblotted onto Nitrocellulose membranes (BIO-RAD). The membranes were blocked in PBS containing 5% skimmed milk and 0.05% Tween 20. Human β1 integrin subunit was detected with rabbit polyclonal antibody (Santa Cruz Biotechnology), β-actin with rabbit polyclonal antibody (Abcam). Immunodetection was accomplished using the enhanced chemiluminescence kit (ECL Kit, Pierce), then films were scanned and protein bands quantitated using the GelImage system (Pharmacia LKB). To quantify the densitometric scans, the background was subtracted and the area for each protein peak was determined.

Adhesion assay. Wells of F8 Maxisorp loose Nunc-Immuno™ modules (Nunc™ brand products) were coated with 50 μl of fibronectin, laminin or collagen type I used at 10 μg/ml in TBS (0.02 M Tris/HCl, 0.15 M NaCl, pH 7.6). Proteins were allowed to bind for 2 h at 37°C before the wells were rinsed twice with TBS and blocked for 1.5 h at 37°C in a humidified 5% CO_2 atmosphere with 200 μl of 1% heat-denatured BSA in TBS, pH 7.5, containing 0.1 mM $CaCl_2$. Cells were harvested and added to the wells at 1.5×10^5 cells/0.15 ml of DMEM for 1.5 h at 37°C in 5% CO_2 atmosphere. Cells that did not adhere were removed by gentle washing with TBS containing 0.1 mM $CaCl_2$, pH 7.5. The total cell-associated protein was determined by dissolving the attached cells directly in the micro-titer wells with 200 μl of Pierce BCA (bicinchonImic acid) protein assay reagent. The modules were incubated for 30 min at 37°C, and the absorbance of each well was determined at 562 nm with a microplate reader (BioKinetics Reader EL340, Bio-Tek Instruments).

Chemoinvasion assay. Invasion assays were conducted on polycarbonate filters, 8 μm (Transwell® Costar®). The filters were coated with basement membrane Matrigel™ (25 μg/filter). The matrigel was spread on to the filters, dried under a hood, and reconstituted with serum-free medium. Cells were harvested with trypsin/EDTA and diluted, depending upon the type of cells tested, in either MEM-α or DMEM with 0.1% BSA to the final density of 2×10^6 cells/ml, and 50 μl of the cell suspension was added to the upper chamber. Conditioned medium was obtained by incubating mouse fibroblasts (3T3) for 24 h in serum-free medium in the presence of ascorbate (50 mg/l). This medium was used as a source of chemoattractants and placed in the lower compartment of the chambers. The assembled chambers were incubated for 6 h at 37°C and 5% CO_2. Then, the cells on the upper surface of the filter were completely removed by wiping with a cotton swab. The filters were fixed in methanol, cells were stained with Mayer's hematoxylin and eosin, and counted from the lower surface of the filter.

Data analysis. All values are expressed as mean ± S.D. and were compared with controls. Significant difference was taken for P values less than 0.05.

RESULTS AND DISCUSSION

Invasion and metastasis are important determinants in the progression of cancer, and the motility of cancer cells is dependent on their interactions with the microenvironment (Liotta & Kohn 2001; Quaranta, 2002). The expression levels of various integrins are up-regulated during cancer progression and the altered cell-ECM interactions are considered important in the invasion and metastasis of tumor cells (Quaranta, 2002). Therefore, in preliminary experiments an analysis of surface membrane integrin expression was performed by flow cytometry in a variety of human colon malignant cell lines. The β1 subfamily of integrins plays a crucial role in cell adhesion

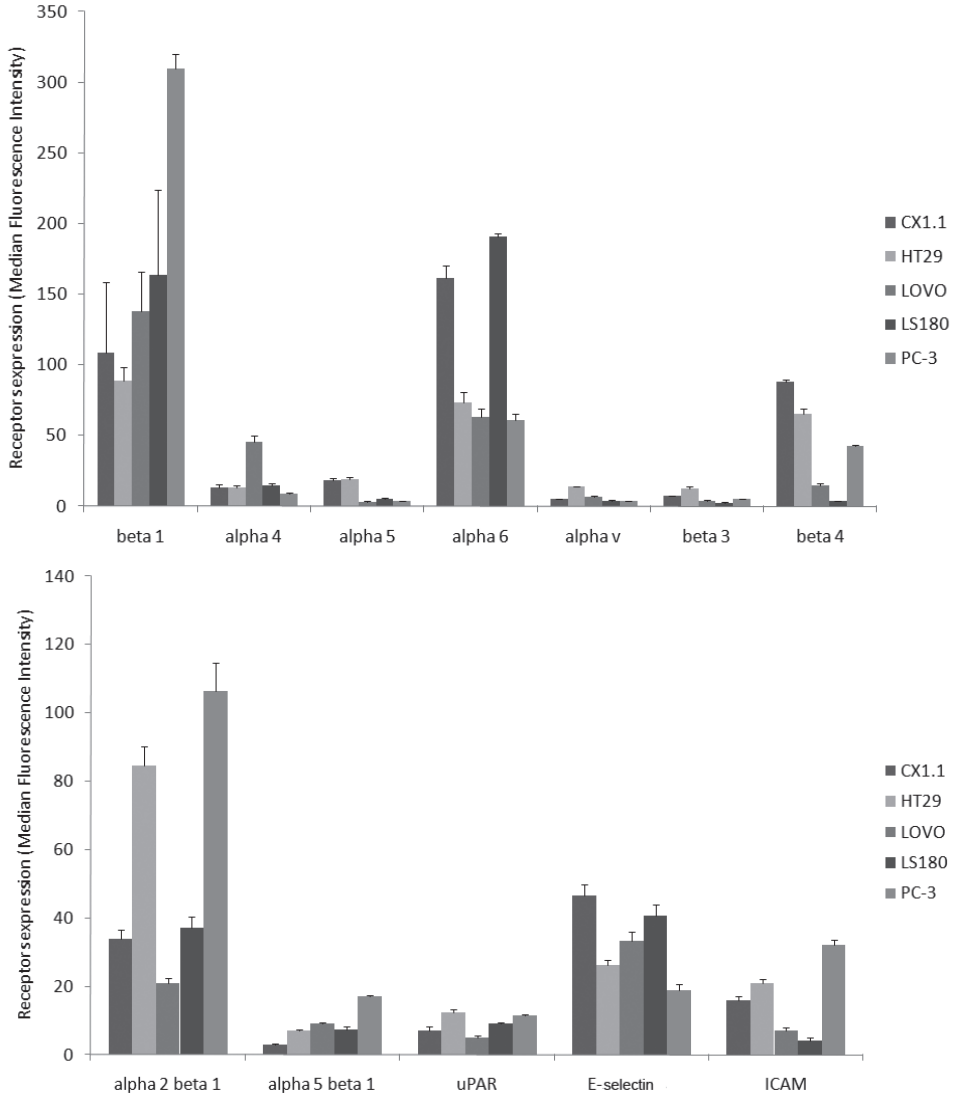


Figure 1. Cell membrane expression of selected integrin subunits and intact adhesive receptors in human cancer cells
Colon carcinoma cell lines (CX1.1, HT29, LOVO, LS180) were stained with monoclonal antibodies to α and β subunits of integrins **(A)** or specific to intact α2β1 and α5β1 as well as uPAR, E-selectin and ICAM **(B)**. In parallel, prostate cancer cells PC-3 were analyzed. Fluorescence intensity of the staining was measured with a FACScan flow cytometer and expressed as Median Fluorescence Intensity.

and invasiveness (Simon-Assmann *et al.,* 1994; Yao *et al.,* 2007). Because the β1 integrin subfamily plays a crucial role in early stages of angiogenesis and invasiveness of cancer cells (Giannelli *et al.,* 2002; Zeng *et al.,* 2006), we also characterized the cell lines with respect to β1 integrin subunit expression. There was high expression of β1 integrin subunit (Fig. 1A), which appeared to be mostly in a complex with α2 forming a primary collagen receptor α2β1. The high expression of α2β1 in human colon carcinoma cells was then confirmed by FACS analysis using monoclonal antibody specific to the intact α2β1 receptor (Fig. 1B).

All human malignant colon cells (CX1.1, HT29, LOVO and LS180) and the prostate PC-3 cells showed high expression of α6. Since the expression of β4 integrin subunit was also increased (CX1.1, HT29, PC-3), α6β4 is likely a major integrin on these cells (Fig. 1A). The ligand of integrin α6β4 is laminin-5, a major structural component of the basement membrane of epithelial tissues (Jones *et al.,* 1998; Borradori & Sonnenberg, 1999). Indeed, the binding of α6β4 integrin and laminin-5 is prerequisite to adhesion, migration, and morphogenetic events of some epithelial cells. Thus, the α6β4 integrin is strongly involved in tumor cell invasion (Lohi, 2001; Mercurio *et al.,* 2001). E-selectin and uPAR are also implicated in tumor cell metastasis (Felding-Habermann *et al.,* 1992; Kruskal *et al.,* 2007; Dass *et al.,* 2008; Kielosto *et al.,* 2009). This is why we decided to check the level of these proteins in human malignant colon and prostate cancer cells (Fig. 1A, 1B). The colon carcinoma cell lines expressed similar and substantial levels of E-selectin and uPAR (Fig. 1B).

Previous studies showed that poorly and highly liver-metastatic colon carcinoma cell lines showed various patterns of adhesion to various substrates and different integrin-mediated adhesion to extracellular matrix (Haier *et al.,* 1998). Adhesion of carcinoma cells was mediated by different integrins depending on the ECM components. Some of these receptors, for example α5β1, were significantly associated with the carcinogenesis and differentiation of colorectal carcinomas, but not with their lymph node metastasis, and the expression level was sug-

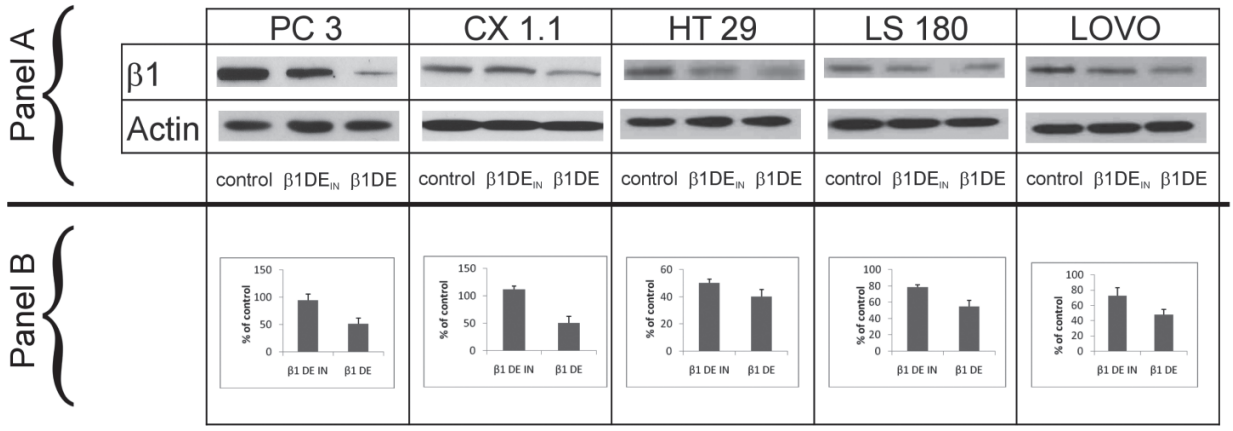
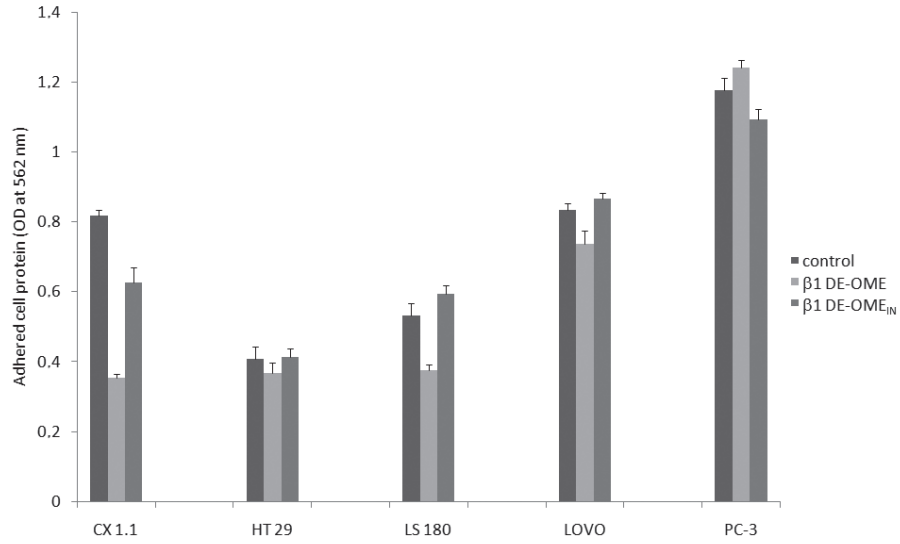
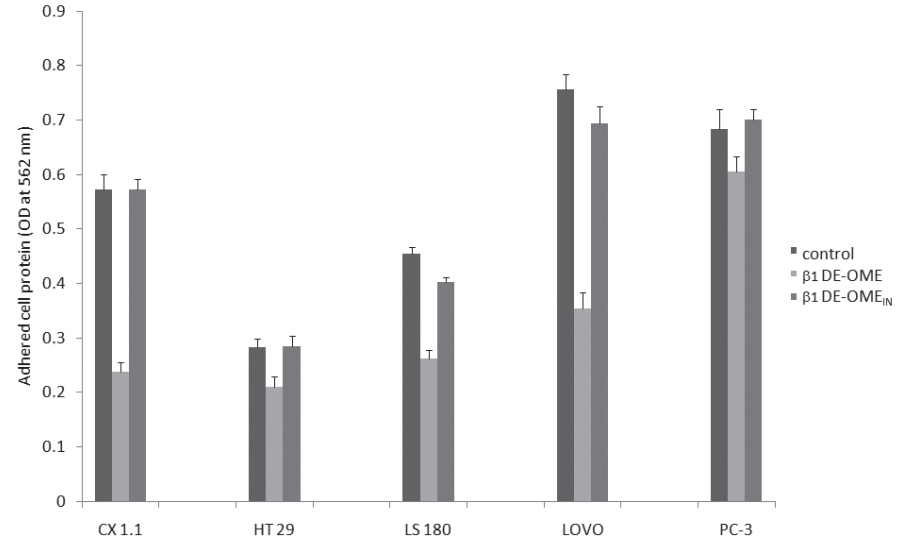


Figure 2. Effect of DNAzyme (β1DE) on β1 integrin subunit level in human colon carcinoma and prostate cancer cells
Panel A shows expression of β1 integrin subunit in PC-3, CX1.1, HT29, LS180, and LOVO analyzed by Western blotting. Cells were treated with nonactive or active DNAzyme, lyzed, proteins were separated by SDS/PAGE, transfered onto nitrocellulose membrane and stained with specific anti-β1 antibodies (Panel A). Then, the membranes were scanned and levels of β1 integrin subunit expressed as mean ± S.D. for 2–5 independent experiments (**Panel B**).





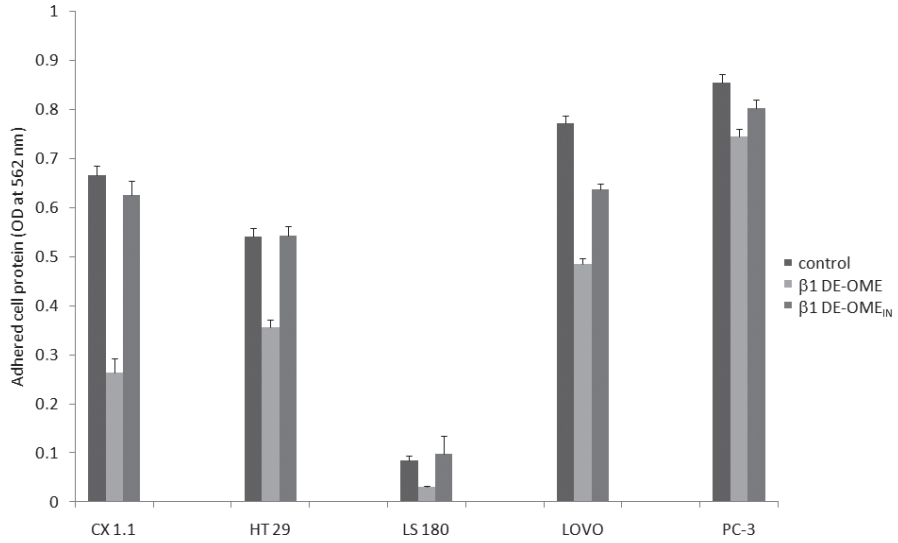


Figure 3. Effect of DNAzyme (β1DE) on adhesion of human colon and prostate cancer cells
Adhesion of CX1.1, HT29, LS180, LOVO cells after incubation with 0.5 μM of β1DE-OMe for 24 h was evaluated using plastic wells coated with fibronectin **(A)**, laminin **(B)** or collagen type I **(C)**. The adhesion, relative to that of untreated cells or cells treated with inactive DNAzyme, was determined based on the measurement of cell protein recovered after solubilization by NaOH of cells adhering to plastic wells and determined with BCA reagent.

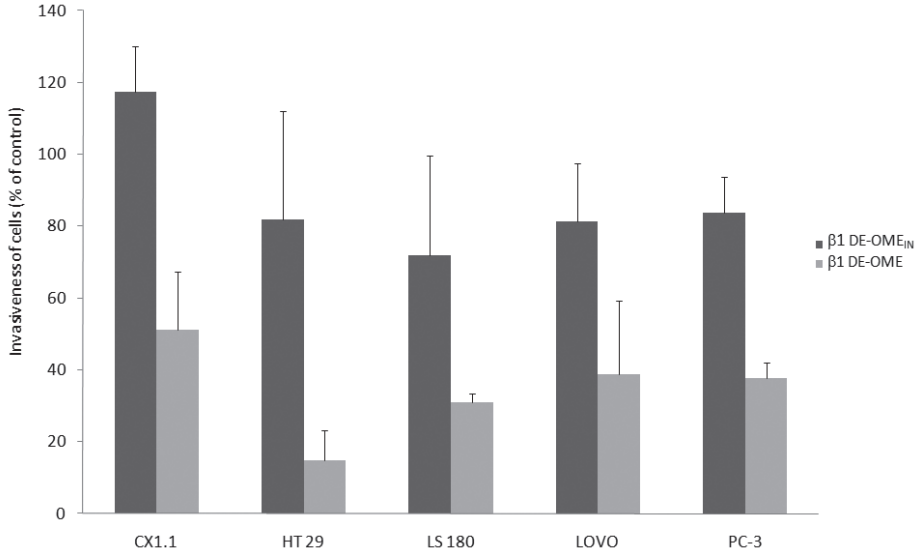


Figure 4. Effect of DNAzyme (β1DE) on invasive properties of human colon carcinoma and prostate cancer cells
CX1.1, HT29, LS180, and LOVO cells were treated with inactive or active DNAzyme to β1 mRNA, allowed to invade Matrigel™ and migrate into the lower part of the filter. Cells on the lower side of the filter were counted and their number was expressed in relation to control cells. (Cells treated with lipofectamine only).

gested to be a valuable indicator for the carcinogenesis and progression of colorectal carcinomas. Because of the great involvement of integrins in cancer metastasis, various potential strategies for inhibiting these receptors have been developed, for example specific antibodies (Chen *et al.,* 2008), receptor antagonists (Qian *et al.,* 2005) or disintegrins (Oliva *et al.,* 2007). The β1 integrin subfamily, particularly α1β1, α2β1, α4β1, α5β1, α6β1 and αvβ1, have been reported to be the most important ones for the interaction of colon carcinoma cells with ECM in the host organs for organ-specific metastasis (Sawhney *et al.,* 2002). To use the β1 subfamily as an anticancer therapy target, inhibitory antibody (Park *et al.,* 2006), nonpeptidic antagonist (Maglott *et al.,* 2006) or anti-tumor protein (Sabherwal *et al.,* 2006) have been examined. In the following experiments we attempted to evaluate the invasiveness of several colon carcinoma cell lines and a prostate cancer cell line after treatment with the DNAzyme to β1 mRNA. In our recent work we found DNAzyme approaches very efficient in inhibition of integrin expression in endothelial cells (Cieslak *et al.,* 2002). The DNAzyme used in this work was previously characterized in terms of its specificity, cellular stability and the mechanism by which it blocked the β1 integrin subunit synthesis (Cieslak *et al.,* 2002). The structural and catalytic properties of this DNAzyme were described in detail in previous work (Cieslak *et al.,* 2003). To reduce β1 subunit expression, colon carcinoma cells and prostate cancer cells were treated with 0.5 μM β1DE-OME for 24 h and tested by Western blotting, also in

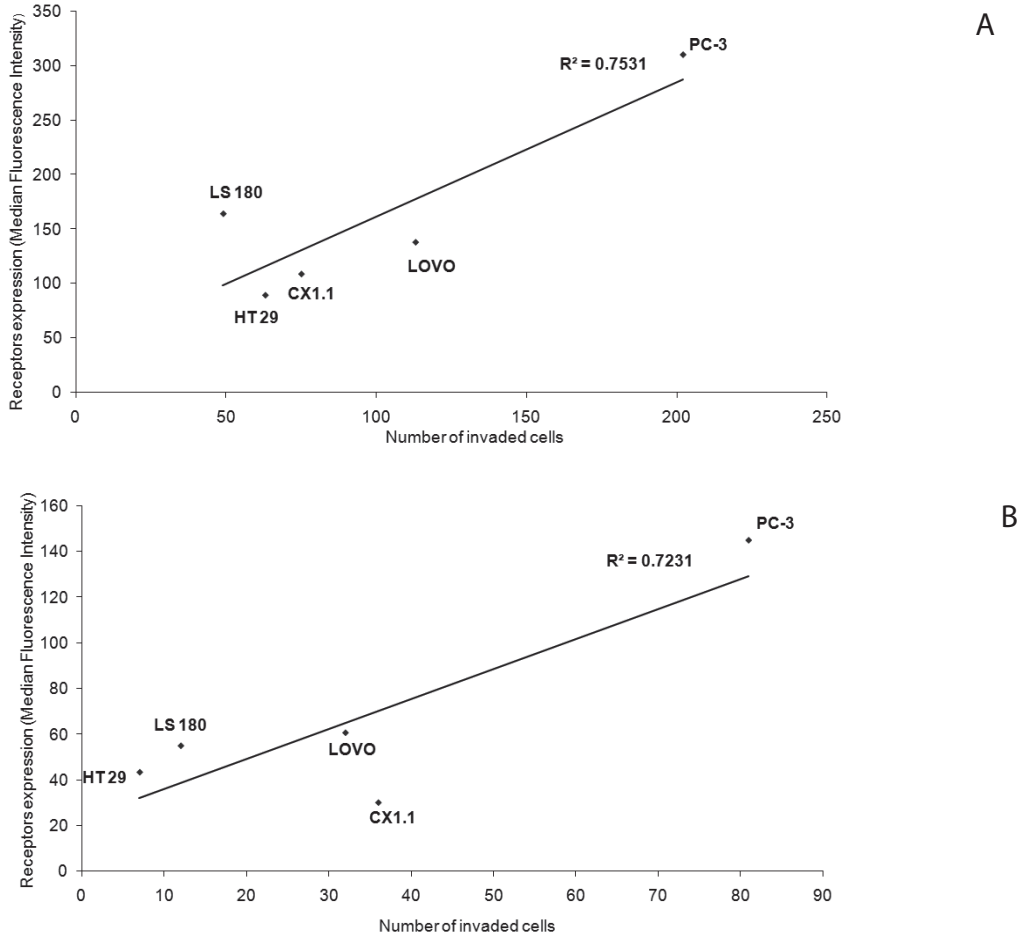


Figure 5. Correlation of invasiveness of cancer cells with their expression of β1 integrin
Invasiveness was evaluated by counting cells on polycarbonate filters covered with Matrigel™ while expression of β1 integrin subunit was evaluated by FACS and expressed as *Median Fluorescence* Intensity. Cells were tested before **(A)** and after **(B)** treatment with β1DE.

adhesion and invasiveness assays. Untreated cells or cells incubated with an inactive DNAzyme under the same conditions served as controls. The Western blot analysis revealed that β1DE-OME downregulated the expression of the β1 integrin subunit at the protein level (Fig. 2A). The highest inhibition was observed in the CX1.1 and PC-3 cell lines (Fig. 2B). As shown in Fig. 3, treatment of colon carcinoma cells with β1DE-OME partly inhibited cell adhesion to fibronectin (Fig. 3A), laminin (Fig. 3B), and collagen (Fig. 3C). CX1.1, HT29, LOVO, and LS180 cells showed similarly reduced adhesive properties towards all three proteins. Among the colon carcinoma cells, CX1.1 cells appeared to be the most sensitive to β1DE-OME, showing adhesion to all three proteins decreased by approx. 60%. It is noteworthy that β1DE-OME hardly affected the adhesion of PC-3 cells to fibronectin (Fig. 3A).

Then we used the *Transwell® migration* assay through a polycarbonate membrane coated with Matrigel to compare the effect of the DNAzyme to β1 mRNA on the invasion of colon carcinoma cell lines and the highly invasive PC-3 cells. In the invasion assay the culture medium obtained after treatment of 3T3 cells with ascorbate, and thus enriched in growth factors, was used as the source of chemotactic migratory stimuli. The downregulation of β1 integrins in colon carcinoma cells, particularly CX1.1, HT29, decreased the number of invading cells by 49% and 85%, respectively (Fig. 4). Also LS180 (minimally invasive colon carcinoma cells) and LOVO (highly invasive colon carcinoma cells), as well as PC-3 (highly invasive prostate cancer cells) showed significantly reduced invasiveness upon treatment with the DNAzyme to β1 mRNA. Interestingly, the invasiveness of PC-3 cells (Fig. 4) was much more sensitive to β1DE-OME treatment than their adhesive properties (Fig. 3A, B, C). We also found that the invasiveness of cancer cells was highly correlated with the expression of β1 integrin subunit both before addition of the inhibitory DNAzyme (Fig. 5A; $R^2 = 0.7531$) and afterwards (Fig. 5B; $R^2 = 0.7231$).

These studies provide direct evidence that blocking β1 integrin subunit synthesis by specific DNAzyme inhibited the adhesion and invasion of the carcinoma cell lines tested. These data confirme previous studies showing a central role for β1 integrin in cancer cell motility and further demonstrate that DNAzymes can be used to investigate the role of β1 integrin subunits in the invasiveness of malignant cells *in vitro*.

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