Original article:

A Role for Integrin αvβ3 in Invasion Activity of Highly Metastatic Syrian Hamster Fibroblasts

G. E. Morozevich, N. E. Kozlova, A. E. Berman[*]

Institute of Biomedical Chemistry by V.N.Orekhovich, Moscow, Russia, Phone: (7) 095 2465072, Fax: (7) 095 245 0857, E-mail: berman@ibmc.msk.ru, ([*]corresponding author)

ABSTRACT

The expression of extracellular matrix (ECM) specific receptors, integrins, was investigated in two closely related strains of oncogenically transformed fibroblasts drastically differing in spontaneous metastasizing. The highly metastatic cells (HM) were shown to express in their surface membranes much higher levels of collagen/vitronectin-specific integrin αvβ3, as compared to their lowly metastatic counterparts (LM). Inhibition of the αvβ3-mediated signal transduction strongly reduced the *in vitro* invasiveness of HM, whereas stimulation of αvβ3 signaling markedly augmented their invasive activity. The data obtained provide a direct evidence for implication of αvβ3 integrin in invasive phenotype of the oncotransformed fibroblasts.

Keywords: Fibroblasts, oncotransformation, metastasis, invasion, integrins

INTRODUCTION

Tumor cell invasion and metastasis is a multistep process involving numerous cell-cell and cell-extracellular matrix associations. Cell interaction with the matrix proteins is mediated through cell surface receptors, of which the best studied are integrins. They are composed of two non-covalently associated α and β subunits and the combination of 8 β subunits with at least 14 α subunits generates a heterogeneous family of over 20 heterodimers (Eble 1997; Humphries 2000). It was initially thought that integrins function merely as linkers between the cells and the ECM, but in recent years it has become evident that integrins can transduce signals controlling various aspects of cell behavior, such as migration, differentiation and apoptosis (Brakebusch et al. 2002; Berman et al. 2003).

Both of these functions of integrins suggest their involvement in the mechanisms of tumor growth and progression (Heino 1996; Hood and Cheresh 2002). But the data from various reports appear to be conflicting. In particular, a positive correlation, which was found in some studies between the expression of distinct integrins and metastatic capacity of tumor cells has not been observed in other investigations (Keely et al. 1998; Berman and Kozlova 2000; Hood and Cheresh 2002).

One reason that might account for these discrepancies is that the majority of published studies have been conducted on epithelial cells which are characterized by a high diversity of integrins. Any matrix protein can bind more than one receptor and individual integrins can recognize more than one matrix protein. This implies that

attachment of a cell to a substrate would remain unchanged if reduced expression of one integrin is compensated by enhanced expression of another receptor having the same substrate binding specificity. Likewise, reduced signal transduction, caused by a decreased expression of one integrin, may be compensated by another receptor, generating the similar signal. On the other hand, two integrins that bind the same ligand can induce distinct cellular responses (Heino 1996; Berman et al. 2003). Therefore, availability of cell lines, differing in metastatic activity while exposing limited patterns of integrins, will facilitate the definition of the role of a particular receptor.

We have previously shown that two strains of Rous sarcoma virus transformed Syrian hamster fibroblasts, differing in spontaneous metastasizing, have a restricted spectrum of integrins exposed on their surface membranes (Kozlova et al. 1997). In this study we have shown for the first time that invasive phenotype of the embryo fibroblasts with high metastatic activity may be accounted for by increased expression and signaling of the collagen/vitronectin-specific integrin $\alpha_v\beta_3$.

MATERIALS AND METHODS

Chemicals
Polyclonal antibodies against the cytodomain of $\alpha5$ integrin subunit and monoconal antibody LM609 to the $\alpha v\beta3$ dimer were purchased from Chemicon (USA). Matrigel, oligopeptides Gly-Arg-Gly-Asp-Ser (GRGDS) and Gly-Arg-Glu-Ser (GRES) as well as N-hydroxysuccinimidobiotin amidocaproate (NHS-biotin) were from Sigma (USA). Oligonucleotides were synthesized by Syntol (Moscow). Reagents for reverse transcription and polymerase chain reaction (RT-PCR) were obtained from Invitrogen (USA).

Cells
The parental strain of Rous sarcoma virus (RSV) transformed Syrian hamster fibroblasts with a low level of spontaneous metastasizing (lowly metastatic cells, LM) and its highly metastatic derivative (HM), were obtained from Russian Cancer Center (Moscow). The cells were propagated in DMEM supplemented with 5% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

Cell adhesion
Cell adhesion to ECM proteins was assayed as described (Berman et al. 1993). 96-well microtiter plates were coated with 10 µg/ml substrate proteins, and free sites were blocked with 5 mg/ml bovine serum protein (BSA). Cells ($7x10^4$ in 100 µl of serum free DMEM, containing 2.5 mg/ml BSA) were seeded in the plates and incubated at 37^0C for 1 hr. Non-adherent cells were washed off, and the number of adhered cells was assessed in a hexosaminidase reaction (Erokhina et al. 1994).

Biotinylation
Biotinylation of cell surface proteins, immunoprecipitation of integrins, electrophoresis, and Western-blotting were performed as described (Kozlova et al. 1997). Cells ($1x10^7$) in PBS (1 ml) were biotinylated with NHS-biotin (100 µg/ml, 30 min., 20^0), washed and lysed in extraction buffer (25 mM tris-HCl, pH 7.5, containing 100 mM octylglucoside, 2.5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 10^{-3} M phenylmethanesulfonic acid, and 10 µg/ml of leupeptin). After centrifugation (15000 g, 20 min.) the biotin content in the extracts was determined by ELISA with avidin-peroxidase conjugate. Cell extracts (1 ml) of equal biotin contents were incubated with 25 µl of anti-integrin antibodies overnight at 4^0C and immunoprecipitates were bound to Protein A-agarose. The precipitates were heat-denatured under non-reducing conditions, separated by 7.5% SDS-PAGE, and transferred to nitrocellulose membrane. Blots were

developed with Enhanced Chemiluminescence System (ECL; Amersham, UK)

In vitro invasion

In vitro invasion was assayed in transwells (Costar, membrane diameter 6.5-mm, pore size 8 μm). The upper chamber was filled with 50 μl of a solution of Matrigel (3 mg/ml) in DMEM and incubated for 2 hr at 37°C to let the gel forms. Cells (2×10^4) in 200-300 μl of the same medium containing 0.5% fetal serum were seeded on the gel, and the lower chamber was filled with 1 ml of the medium. The wells were incubated at 37°C for 24 hr, and then the number of the cells migrated into the lower chamber was determined. In experiments with an RGD containing peptide (Gly-Arg-Gly-Asp-Ser), control peptide Arg-Gly-Glu-Ser (RGE peptide) (500 μg/ml each), cells were incubated with these agents for 30 min. and then placed in the top chamber for the invasion assay.

Stimulation of integrin signaling

Plastic dishes were coated with 25 μg/ml rabbit anti–mouse IgG at 37^0C for 2 hr, followed by blocking with 1% heat-denatured BSA at 37^0C for 1 hr. Then, anti-αvβ3 antibody LM 609 or mouse IgG (each 10 μg/ml) were allowed to bind the immobilized anti–mouse IgG for 14 hr at 4^0C. Subconfluent HM fibroblasts were suspended in serum free DMEM and allowed to attach to antibody-coated dishes for 4 hr at 37^0C. Cells were then processed for in vitro invasion assay as described above.

Reverse transcription-polymerase chain reaction (RT-PCR)

Isolation of total cellular RNA and RT were described earlier (Kozlova et al. 2001). As was evidenced by agarose gel electrophoresis no visible traces of high molecular DNA were found in the RNA preparations. Primer sequences for PCR were the following: αv subunit: 5'-GTTGGGAGATTAGACAGAGGA-3' (sense), 5'-CAAAACAGCCAGTAGCAACAA-3' (antisense); β-actin: 5'-GTGGGGCGCCCCAGGCACCA-3' (sense), 5'-CTCCTTAATGTCACGCACGATTTC-3' (antisense). Denaturing was performed at 94^0C, elongation at 72^0C, annealing at 56^0C (for αv) and 60^0C (for β-actin) (internal standard). The optimal numbers of PCR cycles were 40 for αv and 22 for β-actin. The primers used for cDNA amplification are the sequences located at different exons of the respective genes, separated by one or more introns. Therefore, if genomic DNA contaminations were present in RT preparations, the PCR products amplified from these DNA would be of much higher molecular sizes than those synthesized from the target cDNA. In our experiments, only bands with the expected sizes, corresponding to target cDNAs, were seen in agarose gel electrophoresis.

Statistical analysis

Statistical analysis was carried out using Student' s *t*-test. Differences between groups with $p<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

We first addressed the question of whether there are any differences between LM and HM cells in their adhesion to the matrix proteins. As seen in Fig.1, both cell types demonstrated virtually equal attachment to native type I collagen, fibronectin and laminin. In contrast to these substrates, heat-denatured type I collagen appeared to support the attachment of HM fibroblasts much stronger than of LM cells.
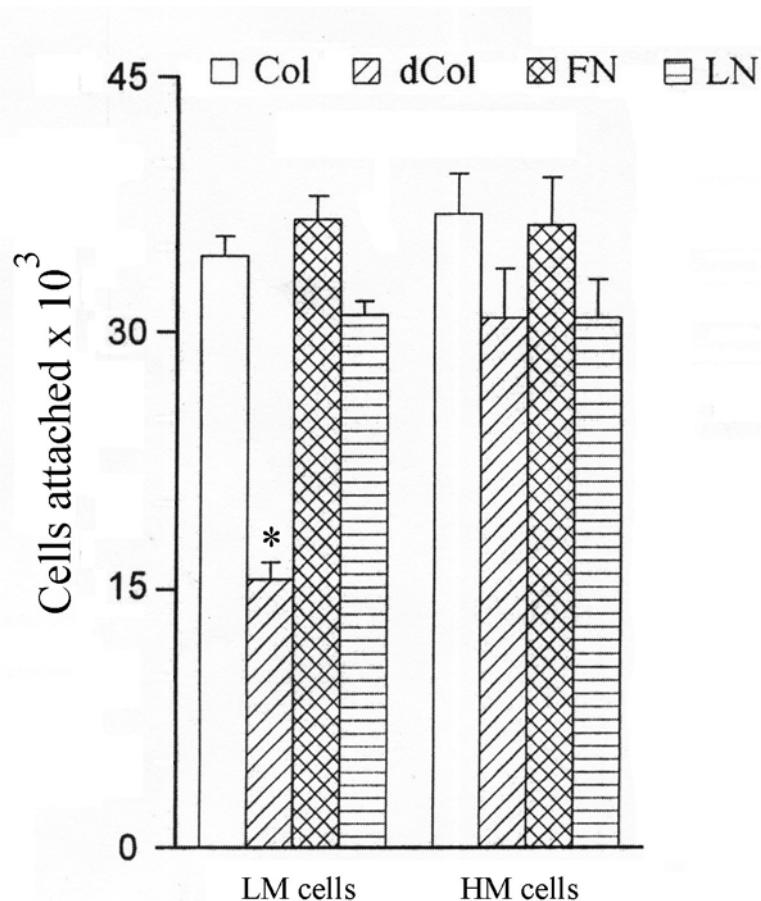
Figure 1: Adhesion of lowly and highly metastatic fibroblasts (LM and HM resp.) to matrix proteins. Cell attachment to matrix proteins was quantified as described in "Materials and methods". Col, type I native collagen; dCol, type I denatured collagen; FN, fibronectin; LN, laminin. Data are mean ± s.e.m. of 3 experiments, each performed in triplicate. *p< 0.01 compared with HM cells adhered to dCol.

Denatured collagen is known to be a specific adhesion substrate for $\alpha v \beta 3$ integrin (Eble 1997). Therefore, the observed differences in adhesive properties suggested that the expression of $\alpha v \beta 3$ could be altered in HM cells.

To test this suggestion, the LM and HM fibroblasts were compared for their cell surface expression of integrins. Western bloting data, presented in Fig. 2a, demonstrate that the HM fibroblasts are much more active in surface expression of

the collagen/vitronectin-specific $\alpha v \beta 3$ receptor. In contrast, HM cells expressed markedly lower amount of $\alpha 5 \beta 1$ integrin, the fibronectin-specific receptor, than LM fibroblasts. These data corroborate the results of adhesion assay.

Data on cell surface expression of integrins were supported by RT-PCR analysis that revealed the reduced αv mRNA in LM compared with HM cells (Fig. 2b).
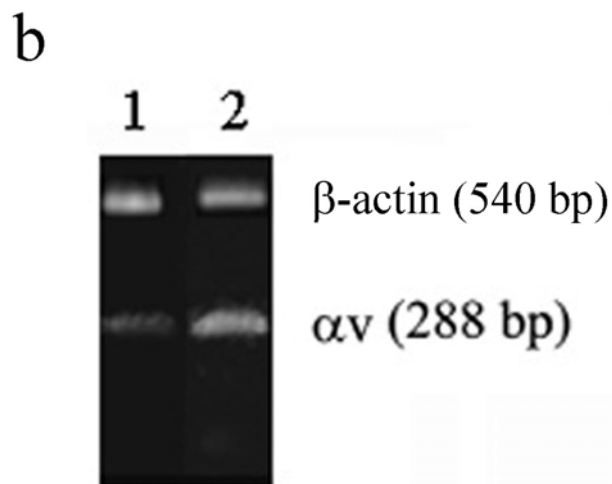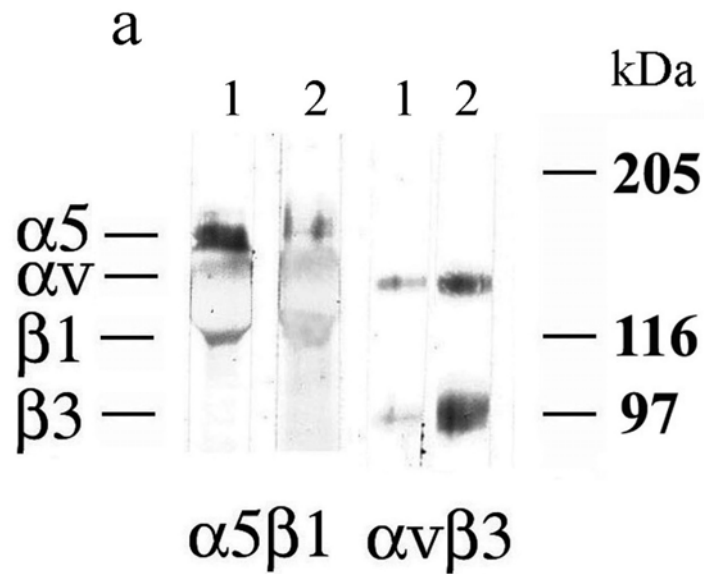
Figure 2: Integrin expression in lowly metastatic (1) and highly metastatic (2) fibroblasts. (a) Cell surface expression of integrins. Biotinylated cell surface proteins (see 'Materials and Methods') were precipitated with anti-integrin antibodies, separated in PAGE and transferred onto a nitrocellulose membrane. Blots were processed as indicated in "Materials and Methods. Right, sizes of molecular weight markers are indicated; left, positions of respective integrin subunits are indicated. (b) RT-PCR analysis of expression of αv mRNA. Expression of β-actin mRNA was used as an internal control. Right, sizes of respective PCR products are indicated.

Invasiveness of lowly and highly metastatic fibroblasts

The initial metastasis stages involve the destruction of surrounding tissue by tumor cells and their migration (invasion) from the primary loci across the basement membrane (Brakebusch et al. 2002; Egeblad and Werb 2002). An *in vitro* model reproducing this process is cell penetration and destruction of a gel formed by gelation of matrix proteins, Matrigel (Heino 1996).
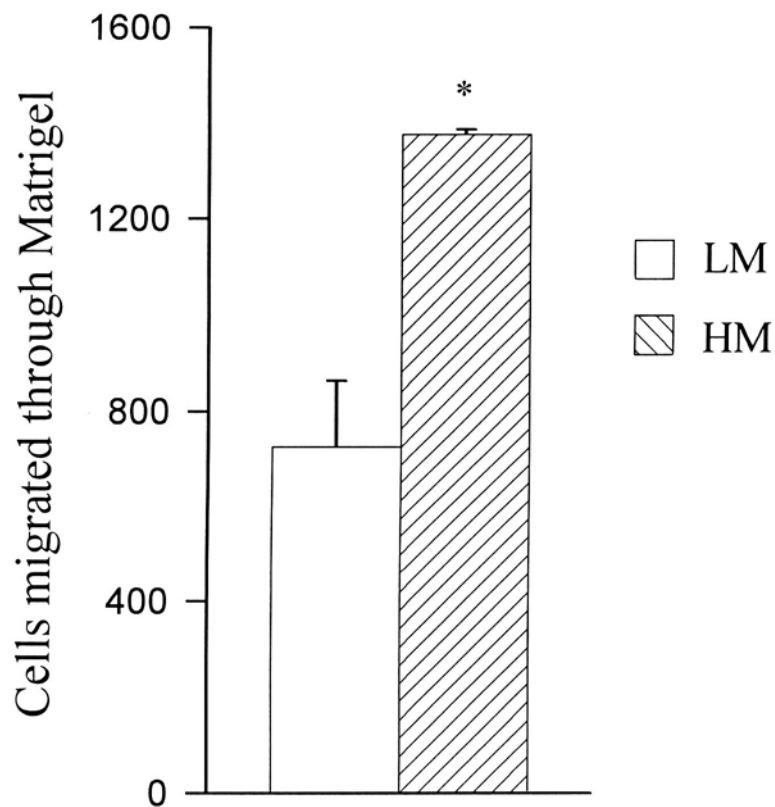
Figure 3: *In vitro* invasion of lowly and highly metastatic fibroblasts. $2 \cdot 10^4$ cells were plated into the upper chamber of a transwell, incubated 24 hr at 37^0C and the number of cells in the lower chamber was determined. Data are mean ± s.e.m. of three independent experiments. * *p* < 0.05 relative to LM.

Figure 3 demonstrates that the highly metastatic fibroblasts are much more active in invasion through Matrigel compared to parent lowly metastatic cells.

We next addressed whether elevated expression of the αvβ3 integrin in highly metastatic cells is associated with their invasion phenotype. We explored two approaches. First, we assessed the effect of an RGD containing peptide known to antagonize integrin mediated signaling (Ruoslahti 1996) on invasiveness of RSV-transformed fibroblasts.

Figure 4 demonstrates that treatment of HM fibroblasts with the RGD-containig peptide resulted in a 10-fold decrease of their invasion through Matrigel while the control RGE peptide was ineffective. In contrast, treatment of LM fibroblasts with the RGD peptide had an insignificant effect on invasion. As seen in Fig. 2, LM cells are active in the expression of α5β1 integrin, unlike the HM fibroblasts exhibiting a high level of αvβ3 expression. Both of these receptors are RGD-dependent and can be modulated by RGD peptides (Brassard et al. 1999). However, only the αvβ3-expressing fibroblasts reduced their invasive activity upon treatment with the RGD peptide. This finding emphasizes an important role of the αvβ3 integrin in invasiveness of HM fibroblasts. Lack of this integrin on LM cells is likely to make them resistant to the RGD peptide.
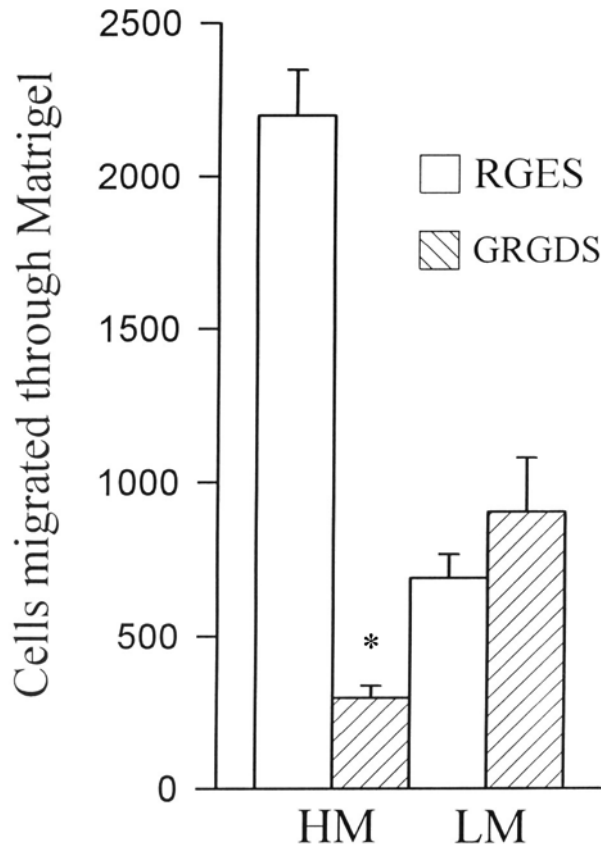
110

Figure 4: Effect of RGD-peptide on invasiveness of highly and lowly metastatic fibroblasts. $2 \cdot 10^4$ cells treated with RGES or GRGDS (see "Materials and Methods") were plated into the upper chamber of a transwell, incubated 24 hr at 37^0C and the number of cells migrated into the lower chamber was determined. Data are mean $\pm$ s.e.m. of three independent experiments. * $p < 0.01$ relative to HM treated with RGES.

Second approach is based on the ability of integrins, ligated by anti-integrin antibodies, to mediate signal transduction and thereby modulate cell behaviour (Leavesley et al. 1993).

Table 1: Activation of $\alpha v \beta 3$ integrin signaling stimulates the *in vitro* invasion of highly metastatic cells.

Treatment	Invasion, % Control
None (Control)	100
Mouse IgG	110 ± 11
Anti-$\alpha v \beta 3$	159 ± 16 *

Cells were treated with mouse IgG or anti-$\alpha v \beta 3$ antibody LM 609 and assayed for the *in vitro* invasion as described in "Materials and Methods". Data are mean $\pm$ s.e.m. of three independent experiments. * $p < 0.05$ relative Control and mouse IgG.

To examine whether ligation of αvβ3 influences invasion of tumor cells, HM fibroblasts were plated on a substrate consisting of immobilized mouse IgG or anti-αvβ3 antibodies. Table 1 clearly shows that activation of αvβ3-mediated signaling markedly increased the invasive activity of HM cells whereas treatment with mouse IgG had no effect. This finding confirms the experiments with the RGD peptide.

Data concerning the role of the αvβ3 integrin in malignancy are ambiguous. In some reports, the important role of this receptor in invasion and metastasis was emphasized based on its elevated expression in a number of tumor-derived lines with high metastatic activity: cervical cancer (Chatterjee et al. 2001), hepatocellular carcinoma (Nejjari et al. 2002), etc. These findings corroborate the results demonstrating that the *in vivo* administered αvβ3 antagonists strongly decreased dissemination of metastases and increased survival of animals (Reinmuth et al. 2003.; Kumar 2003). But these observations are not in line with the report that no overexpression of αvβ3 was found in the actively metastasizing stomach carcinoma cells (Yasoshima et al. 1996) and, moreover, re-expression of this receptor in a line of the αvβ3-deficient highly metastatic cells attenuated experimental metastasizing (Danen et al. 1996).

Similar contradictions were reported for expression of α5β1 and α6β4 integrins by tumor cells (Hood and Cheresh 2002; Clezardin 1998; Danen et al. 1996; Yao et al. 1997; Kawashima et al. 2001).

In this study, a strong correlation was documented between the expression of collagen-specific integrin αvβ3, metastatic and invasive activity of oncotransformed fibroblasts and their affinity to denatured collagen. On the other hand, we have recently demonstrated that upon acquisition of multidrug resistance, breast carcinoma cells increased their invasive capacity which was accompanied by a marked down-regulation of the αvβ3 receptor and dramatic up-regulation of integrin α5β1 (Kozlova et al. 2004). One suggestion to reconcile these contradictions is that the acquisition of drug resistance may alter integrin-mediated signal transduction pathways. Another possibility is that during malignant evolution the breast carcinoma cells and oncotransformed fibroblasts were surrounded by different matrix proteins which induced each particular cell type to expose specific integrins on cell surface. Given that different integrins can transduce signals with similar physiological consequences (Boudreau and Jones 1999; Berman et al. 2003), one can assume that α5β1 in breast carcinoma cells and αvβ3 in transformed fibroblasts play a similar role in tumor progression.

In conclusion, the data presented here and our previous results, as well as those reported by other authors, imply that each particular integrin can participate in establishment of particular phenotype depending on extracellular matrix content.

REFERENCES

Berman AE, Kozlova NI. Integrins: structure and functions. *Membr Cell Biol* 2000;13:207-44

Berman A, Morozevich G, Karmansky I, Gleiberman A, Bychkova V. Adhesion of mouse hepatocytes to type I collagen: role of supramolecular forms and effect of proteolytic degradation. *Biochem Biophys Res Commun* 1993;194:351-7

Berman AE, Kozlova NI, Morozevich GE. Integrins: structure and signaling. *Biochemistry (Moscow)* 2003;68:1284-99

Boudreau NJ, Jones PL. Extracellular matrix and integrin signalling: the shape of things to come. *Biochem J* 1999;339:481-8

Brakebusch C, Bouvard D, Stanchi F, Sakai T, Fassler R. Integrins in invasive growth. *J Clin Invest* 2002;109: 999-1006

Brassard DL, Maxwell E, Malkowski M, Nagabhushan TL, Kumar CC, Armstrong L. Integrin alpha(v)beta(3)-mediated activation of apoptosis. *Exp Cell Res* 1999;251:33-45

Chatterjee N, Chatterjee A. Role of alphavbeta3 integrin receptor in the invasive potential of human cervical cancer (SiHa) cells. *J Environ Pathol Toxicol Oncol* 2001;20:211-21

Clezardin P. Recent insights into the role of integrins in cancer metastasis. *Cell Mol Life Sci* 1998;54:541-8

Danen EH, van Kraats AA, Cornelissen IM, Ruiter DJ, van Muijen GN. Integrin beta 3 cDNA transfection into a highly metastatic alpha v beta 3-negative human melanoma cell line inhibits invasion and experimental metastasis. *Biochem Biophys Res Commun* 1996;226:75-81

Eble J A. Integrins – a versetile and old family of cell adhesion molecules. In: *Molecular Biology Intelligence Unit. Integrin–Ligand Interaction* (Eble, J. A., and Kunn, K., eds.) Chapman, and Hall, New York.1997; pp. 1-40

Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* 2002;2:161-74

Erokhina MV, Shtil AA, Shushanov SS, Sidorova TA, Stavrovskaya AA. Partial restoration of the actin cytoskeleton in transformed Syrian hamster fibroblasts selected for low levels of 'typical' multidrug resistance. *FEBS Lett* 1994;341:295-8

Heino J. Biology of tumor cell invasion: interplay of cell adhesion and matrix degradation. *Int J Cancer* 1996;65:717-22

Humphries MJ. Integrin structure. *Biochem Soc Trans* 2000;28:311-39

Hood JD and Cheresh DA. Role of integrins in cell invasion and migration. *Nat Rev Cancer* 2002;2:91-100

Kawashima A, Kawahara E, Tokuda R, Nakanishi I. Tumour necrosis factor-alpha provokes upregulation of alpha2beta1 and alpha5beta1 integrins, and cell migration in OST osteosarcoma cells. *Cell Biol Int* 2001;25:319-29

Keely P, Parise L, Juliano R. Integrins and GTPases in tumour cell growth, motility and invasion. *Trends Cell Biol* 1998; 3:101-6

Kozlova NI, Morozevich GE, Chubukina AN, Berman AE. Integrin alphavbeta3 promotes anchorage-dependent apoptosis in human intestinal carcinoma cells. *Oncogene* 2001;20:4710-7

Kozlova NI, Morozevich GE, Chubukina AN, Shtil AA, Berman AE. Expression of integrins, anchorage dependent apoptosis and invasiveness of multidrug resistant human breast carcinoma cells. *EXCLI J* 2004;3:68-81

Kozlova NI, Morozevich GE, Soloveyva NI, Vinokurova SV, Berman AE. Integrin expression and collagenase activity of RSV-transformed Syrian hamster fibroblasts, differing in spontaneous metastasizing. *Biochem Mol Biol Int*. 1997;43:529-39

Kumar CC. Integrin alpha v beta 3 as a therapeutic target for blocking tumor-induced angiogenesis. *Curr Drug Targets* 2003;4:123-31

Leavesley DI, Schwartz MA, Rosenfeld M, Cheresh DA. Integrin beta 1- and beta 3-mediated endothelial cell migration is triggered through distinct signaling mechanisms. *J Cell Biol* 1993;121:163-70

Nejjari M, Hafdi Z, Gouysse G, Fiorentino M, Beatrix O, Dumortier J, Pourreyron C, Barozzi C, D'errico A, Grigioni WF, Scoazec JY. Expression, regulation, and function of alpha V integrins in hepatocellular carcinoma: an in vivo and in vitro study. *Hepatology* 2002;36:418-26

Reinmuth N, Liu W, Ahmad SA, Fan F, Stoeltzing O, Parikh AA, Bucana CD, Gallick GE, Nickols MA, Westlin WF, Ellis LM. Alpha-v/beta-3 integrin antagonist S247 decreases colon cancer metastasis and angiogenesis and improves survival in mice. *Cancer Res* 2003;63:2079-87

Ruoslahti E. RGD and other recognition sequences for integrins. *Annu Rev Cell Dev Biol* 1996;12:697-715

Yao M, Zhou XD, Zha XL, Shi DR, Fu J, He JY, Lu HF, Tang ZY. Expression of the integrin alpha5 subunit and its mediated cell adhesion in hepatocellular carcinoma. *J Cancer Res Clin Oncol* 1997;123:435-40

Yasoshima T, Denno R, Kawaguchi S, Sato N, Okada Y, Ura H, Kikuchi K, Hirata K. Establishment and characterization of human gastric carcinoma lines with high metastatic potential in the liver: changes in integrin expression associated with the ability to metastasize in the liver of nude mice. *Jpn J Cancer Res* 1996;87:153-60