Original article:

The role of α2β1 integrin in anchorage dependent apoptosis of breast carcinoma and hepatoma cells

N. I. Kozlova[1], G. E. Morozevich[1], N.A. Ushakova[1], M.E. Preobrazhenskaya[1], A.A. Shtil[2], A.E. Berman[1]*

[1]V.N.Orekhovich Institute of Biomedical Chemistry, [2]N. N. Blokhin Cancer Center, Moscow, Russia, Phone: (7) 495 2463345, Fax: (7) 495 245 0857, E-mail: *berman@ibmc.msk.ru* (*corresponding author)

ABSTRACT

The role of collagen specific α2β1 integrin in anchorage dependent apoptosis (anoikis) was investigated. Stimulation of α2β1 signaling with immobilized anti-α2 antibody markedly sensitized human MCF-7 breast carcinoma and HepG2 hepatoma cells to anoikis. Accordingly, down-regulation of α2β1 by α2-specific siRNA decreased the percentage of cells undergoing anoikis. These results for the first time provide direct evidence that α2β1 receptor can transduce the signal to promote death in matrix deprived cells.

Key words: extracellular matrix, integrins, signaling, apoptosis, anoikis

INTRODUCTION

Altered response of tumor cells to environmental stimuli is a pleiotropic phenomenon. Integrins, the receptors that mediate interactions of cells with extracellular matrix, are intensely investigated as key regulators of cell survival. The function of integrins in maintaining cell viability is substantiated by their role in anchorage dependent apoptosis (anoikis) (Frisch and Screaton 2001; Cheresh and Stupack 2002). This type of apoptosis is triggered by cell detachment from matrix (Frisch and Screaton 2001; Frisch and Ruoslahti 1997).

Integrin mediated signaling pathways that regulate cell survival remain poorly understood. Integrins are commonly thought to transduce pro-survival signals when cells interact with matrix. Upon disruption of cell-matrix contacts integrins trigger death signaling (Frisch and Screaton 2001; Frisch

and Ruoslahti 1994). However, in a limited number of studies integrins have been shown to promote cell death (Cheresh and Stupack 2002; Kozlova et.al. 2001; Marco RA et.al 2003). We have shown that vitronectin specific integrin αvβ3 promoted anoikis of colon carcinoma cells (Kozlova et.al. 2001) Moreover, in our experiments the loss of α2β1 collagen specific integrin was associated with higher resistance to anoikis in breast carcinoma cells selected for multidrug resistance (Morozevich et.al. 2006).

Based on these results we set out to explore the role of α2β1 receptor in regulation of cell death. The α2β1 signaling was stimulated by the cross-linking antibody; alternatively, the function of α2β1 was attenuated by genetic down-regulation of α2 subunit. We report that stimulation of α2β1 signaling in two cell lines of different tisssue origin, namely,

breast carcinoma MCF-7 and hepatoma HepG2, promotes anoikis whereas down-regulation of this receptor attenuates anoikis. These results for the first time provide the direct evidence for stimulation of anoikis via α2β1 receptor.

MATERIALS AND METHODS

Cells and reagents

The MCF-7 breast carcinoma and HepG2 hepatoma cell lines (obtained from American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37^0C, $5\%CO_2$ in humidified atmosphere. The reagents were from Sigma Chemical Co., St.Louis, MO unless specified otherwise. Anti-human α2β1 monoclonal antibody (mAb) BHA2.1 and rabbit polyclonal antibodies to human α2 subunit were purchased from Chemicon (Temecula, CA). ICO-53 mAb to human HLA-ABC histocompatibility complex was a gift of A.Baryshnikov (N.N.Blokhin Cancer Center, Moscow). The mAb against α-tubulin and siRNA duplexes specific to α2 subunit and control siRNA were from Santa Cruz Biotech. (Santa Cruz, CA). Reagents for reverse transcription and polymerase chain reaction (RT-PCR) were from Invitrogen (Carlsbad, CA).

siRNA Transfection

The procedure was performed according to the manufacturer's instructions (Santa Cruz Biotech.). Briefly, $1-2x10^5$ cells in 1 ml of antibiotic-free DMEM + 10% FBS were plated into each well of 12-well culture plates (Corning, Big Flats, NY). After reaching ~50% confluence the cells were transfected with 50 nM siRNA (final concentration) for 48 h using siRNA transfection reagent. Then cells were harvested with trypsin/EDTA, washed three times with antibiotic-free DMEM+10% FBS, plated on 12-well plates and incubated in the same medium containing 1 μCi [U-^{14}C]thymidine (Amersham, UK). When the cells reached ~50% confluence, they were repeatedly transfected with respective siRNAs for another 48 h using the same conditions.

Cell Stimulation with Immobilized Antibody

Twenty four well plates (Corning) were coated with 25 μg/ml rabbit anti-mouse polyclonal antibodies (Sigma) in phosphate buffered saline (PBS) for 2 h at 37^0 C followed by blocking with bovine serum albumin (BSA; 10 mg/ml in PBS) at 37^0C for 1 h. The plates were then coated with 1:100 dilutions of control or anti-α2 mAb for 18 h at 4^0C. The separate plates were coated with 200 μg/ml poly-L-lysine (PLL, MP-Biomedicals, Germany) at 4^0C for 18 h. Then plates were washed three times with medium and used for cell plating. Cells at log phase were incubated in complete growth medium contaning [U-^{14}C]thymidine (4 μCi/10^6 cells) for 18 h at 37^0C, washed and harvested in the medium containing 2.5% FBS. Labeled cells were plated on immobilized anti-integrin antibodies or PLL and incubated at 37^0C for 24 h. Then cells were collected and subjected to anoikis.

Induction and Quantitation of Anoikis.

The non-adhesive substrate polyhydroxyethyl metacrylate (poly-HEMA, Sigma) was prepared in 48-well plates as described (McGahon et.al. 1995). Cells treated with siRNA or immobilized antibody and labeled with [U-^{14}C]thymidine were plated on poly-HEMA–coated wells and incubated at 37^0C for 24 h. Anoikis was quantitated as in refs. [McGahon et.al. 1995; A Patki and Lederman 1996; Kozlova et.al. 2001].

RT-PCR

Isolation of total cellular RNA, RT and PCR were performed as described (Kozlova et.al. 2001). The following primers were used to amplify the fragments of human α2 (541 bp) and β-actin (540 bp) cDNAs: α2: 5'-TGGGGTGCAAACAGACAAGG-3' (forward), 5'-GTAGGTCTGCTGGTTCAG-3' (reverse); β-actin (internal standard): 5'-GTGGGGCGCCCCAGGCACCA-3'

(forward), 5'-CTCCTTAATGTCACGCACGATTTC-3' (reverse). In preliminary experiments the reaction conditions were established to find the optimal number of cycles within the exponential range of the yield of PCR products. The PCR products were resolved in 1.5 % agarose gel, scanned and analyzed using ImageJ NIH software.

Western Blotting
The procedure was performed as described in ref. (Kozlova et.al. 1997). Blots were incubated with primary antibodies at indicated dilutions (see Figure Legends) for 1 h at room temperature followed by addition of horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000, 1 h at room temperature). Proteins were detected using Enhanced Chemoluminescence System (Amersham). Scanning densitometry with ImageJ NIH software was used to calculate relative expression of proteins.

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

RESULTS AND DISCUSSION

Stimulation of α2β1 Signaling Potentiates Anoikis of MCF-7 and HepG2 Cells
The idea to implicate α2β1 integrin in promotion of anoikis stemmed from our observation that this receptor was dramatically down-regulated in multidrug resistant MCF-7Dox cells compared with their parental MCF-7 counterparts (Morozevich et.al. 2006). The loss of α2β1 receptor correlated with markedly decreased anoikis in MCF-7Dox cells. These data allowed us to hypothesize that in wild type MCF-7 cells α2β1 integrin might mediate death signaling after the cells lose contact with matrix, and stimulation of this integrin is expected to be pro-apoptotic. Likewise, down-regulation of α2β1 would rescue cells from anoikis.

To test this hypothesis, MCF-7 cells were attached to anti-integrin antibodies immobilized on the solid support. Adhesion to immobilized antibodies is known to cause clustering of surface receptors and trigger signaling via these receptors (Kozlova et.al. 2001; Stromblad et.al. 1996). We have previously found that treatment with anti-β1 antibody promoted anoikis of MCF-7 cells (1.7-fold increase of anoikis compared to cells exposed to anti-HLA antibody or PLL (Morozevich et.al. 2006). Among the β1 family integrins expressed in MCF-7 cells, the α2β1 receptor was found on the surface of 90% cells (Table 1). As shown in Fig. 1, in MCF-7 cells incubated on immobilized anti-α2β1 antibody, anoikis was markedly enhanced (up to 1.8-fold increase compared to cells treated with anti-HLA antibody or PLL). These data implicate the α2β1 receptor in transducing the signal(s) to promote the substrate dependent cell death.

We next sought to expand our observations to an independent model with similar spectrum of β1 family integrins. Table 1 shows that the expression of α2, α3, αv and β1 subunits was close in MCF-7 and HepG2 cell lines. The major β1 integrin expressed on HepG2 cells was α2β1 receptor (~70% positive cells; Table 1). In agreement with the results obtained with MCF-7 cells, stimulation of α2β1 signaling in HepG2 cells increased the percentage of anoikis up to 1.5-fold compared with mock-stimulated cells (Fig. 1). Of note, matrix deprived MCF-7 and HepG2 cells displayed an apoptotic mode of death as determined by the 'laddering' of DNA fragments in agarose gel electrophoresis and an increased number of cells with sub-diploid DNA content (by flow cytometry analysis) (data not shown).
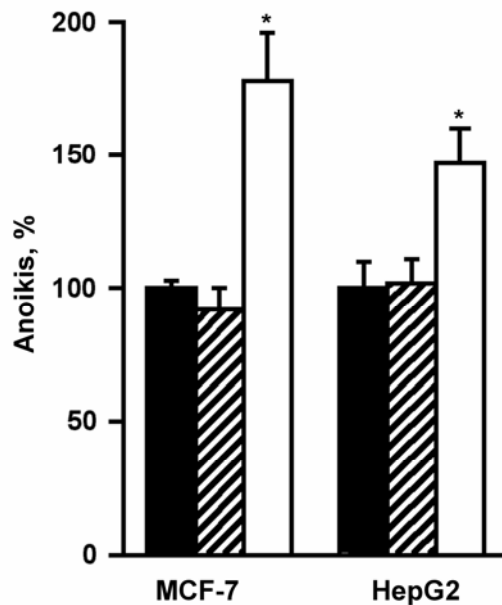
Figure 1: Effect of antibody-induced activation of α2β1 integrin on anoikis in MCF-7 and HepG2 cells. 2.5×10^5 cells labeled with [U-^{14}C]thymidine were resuspended in DMEM containing 2.5% FBS, transferred into 24-well plates coated with ICO-53 anti-HLA mAb (control; closed bars), PLL (hatched bars) or BHA2.1 anti-α2β1 mAb (open bars) and incubated at 37^{0}C for 24 h. Then cells were harvested in DMEM with 2.5% FBS and incubated in 48-well plates coated with poly-HEMA at 37^{0}C for 24 h. After exposure to poly-HEMA, cells were lysed in buffered 0.4% Triton X-100. Intact DNA was pelleted (Kozlova et al., 2001; McGahon et al.,1995). Anoikis was calculated as ratio c.p.m. supernatant/ c.p.m. supernatant + c.p.m. pellet (McGahon et al.,1995). Apoptosis of ICO-53-treated cells was regarded as 100%. Shown are the mean ± s.e.m. of three independent experiments with four replicates in each. *$P < 0.02$ compared to control.

Table 1: Analysis of Integrin Expression in MCF-7 and HepG2 Cells by Flow Cytometry.

Integrin subunit	MCF-7	HepG2
α2	90 ± 1.0	70 ± 6.0
α3	43 ± 6.0	32 ± 3.0
αv	39 ± 4.0	50 ± 2.0
β1	93 ± 1.0	86 ± 2.0

Two hundred thousand cells were resuspended in 50 µl of ice-cold PBS, incubated with integrin subunit-specific antibodies diluted 1:200 for 1 h at 4^{0}C, pelleted and washed twice with PBS. Cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (1:300) for 30 min. at room temperature, washed with PBS and fixed with 1% formaldehyde in PBS. Flow cytometry was performed on FACS Calibur (Becton Dickinson, San Jose, CA) on FL1. Ten thousand events were collected per each sample. Data represent the percent of cells expressing the respective integrin subunit (mean ± s.e.m. of four independent experiments).

Silencing of α2β1 Attenuates Anoikis in MCF-7 and HepG2 Cell Lines

To further substantiate the role of α2β1 receptor in anoikis we used α2-specific siRNA to down-regulate α2 expression. Figure 2 shows that transfection of α2-siRNA markedly decreased the expression of α2 mRNA and protein in both MCF-7 and HepG2 cell lines. Furthermore, down-regulation of α2 expression resulted in statistically significant attenuation of anoikis (Fig. 3). This attenuation was more pronounced in MCF-7 cells compared to HepG2 cells. It is noteworthy that, prior to plating on poly-HEMA, the percentage of dead cells was negligible (<5% after incubation with siRNA), indicating that treatment with siRNAs was not toxic. Thus, together with the data on cell stimulation with anti-α2 antibodies (Fig. 1), the experiments with genetic silencing of α2 prove that α2β1 integrin can promote anoikis at least in two human carcinoma cell lines of different tissue origin.

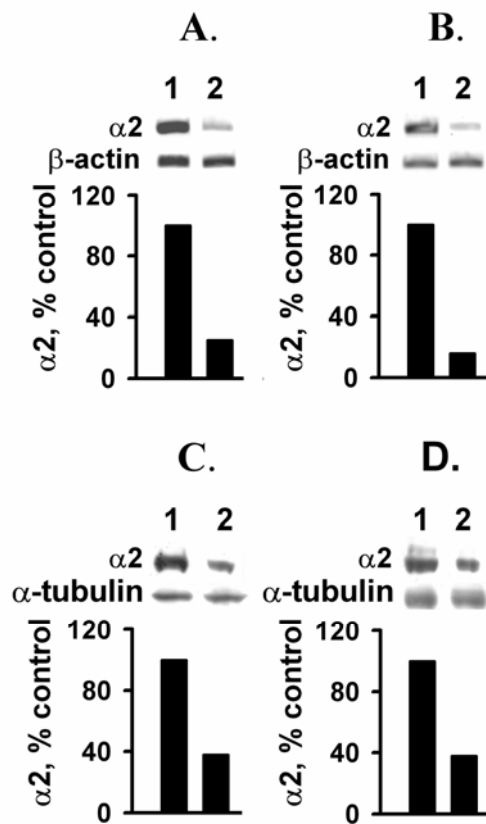**Figure 2:** Silencing of α2 by siRNA. Fifty percent confluent MCF-7 (A, C) or HepG2 (B, D) cells were treated with 50 nM control (1) or α2-specific (2) siRNAs. (A, B), RT-PCR. PCR-amplified fragments of β-actin cDNA and α2-cDNA were resolved in a 1,5% agarose gel, scanned under UV light followed by densitometry. (C, D), Western blottting. Thirty μg of total cellular protein were resolved in SDS-PAGE and blotted onto nitrocellulose. Blots were probed with α-tubulin antibody (1:1000) or α2 antibody (1:500). The Y axis shows the ratio integrin/β-actin (A, B) or integrin/α-tubulin (C, D) in cells treated with respective integrin subunit specific siRNA to that in cells treated with control siRNA (100%).

The role of integrins in cell survival is multifaceted. The bulk of literature provides evidence for the anti-apoptotic role of these receptors. The antagonists of αvβ3 that block its binding to matrix trigger apoptosis of angiogenic cells of chicken embryo (Brooks et al. 1994), endothelial cells grown in a 3-dimensional gel (Kuzuya et.al. 1999), brain tumor cells (Taga et al. 2002) and melanoma (Koistinen et al. 2004). However, much fewer data implicate integrins in pro-apoptotic signaling. We have demonstrated that αvβ3 receptor was capable of stimulating anoikis in colon carcinoma cells (Kozlova et al. 2001). These results were independently confirmed in endothelial cells and osteoclasts (Cheresh and Stupack 2002; Zhao et al. 2005).
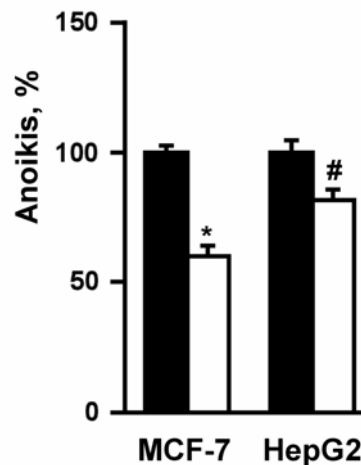

Figure 3: Effects of Integrin Silencing on Anoikis. Cells were treated with control (closed bars) or α2-specific (open bars) siRNAs and labeled with [U-^{14}C]thymidine. Further procedures were as in Fig. 1. Apoptosis of control siRNA treated cells was regarded as 100%. Shown are the mean ± s.e.m. of three independent experiments with four replicates in each. *P < 0.02, $^\#$P < 0.05 compared with control.

As for α2β1, its down-regulation induced apoptosis of MDCK cells grown in a 3-dimensional collagen gel (Saelman et.al. 1995); also, inhibition of α2β1 caused death of colon carcinoma cells adhered on collagen (Buda et al. 2003). In contrast, this study shows the pro-apoptotic role for α2β1 receptor in cells deprived from matrix. One candidate for death signaling downstream of α2β1 engagement can be caspase 8 given that this enzyme was required for another type of cell death, i.e., integrin-mediated apoptosis via unligated β1 family integrins (Cheresh and Stupack 2002). The `atypical' effect of integrins on apoptosis elucidates their involvement in versatile and efficient pathways of the cell survival control. The essence of these pathways is that the same receptor sends a `rescue' signal in a normal physiological situation and shifts it to a `death' signal under abnormal conditions when the death of `homeless' cells would be advantageous for the population.

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