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Role of Integrin αvβ3 in Substrate-Dependent Apoptosis of Human Intestinal Carcinoma Cells

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Abstract—Incubation of human intestinal carcinoma Caco-2 cells in suspension (i.e., in the absence of substrate contacts) leads to massive cell death by apoptosis. Since this type of apoptosis has been referred to as anoikis, we designated these cells as anoikis-positive. However, a minor proportion of Caco-2 cells, designated as anoikis-negative, survived in suspension. Extended incubation of the cells in suspension resulted in the reduction of the number of viable cells. In comparison to the original Caco-2 cell population, the anoikis-negative cells demonstrated markedly decreased levels of expression of integrin αvβ3 on the cell surface and of transcription of the αv subunit gene. Activation of the signaling function of αvβ3 in the original Caco-2 cells led to substantial stimulation of anoikis, while the inhibition of expression of this receptor resulted in better resistance of the cells to anoikis. The data provide the first evidence that αvβ3 integrin can generate apoptosis-stimulating signals.

Key words: integrins, apoptosis, anoikis, extracellular matrix

So-called substrate-dependent apoptosis is one of the forms of apoptotic cell death. It is induced after the disruption of cell contacts with extracellular matrix (substrate). This form of apoptosis has been referred to as anoikis (from a Greek word meaning "homeless") [1-3]. A number of studies indicate that integrins play an important role in apoptosis. Integrins are receptors of the cell membrane that ensure interaction of the cells with matrix proteins and mediate the transduction of substrate-induced signals that control various intracellular reactions. It has been shown that certain integrins in the substrate-bound form transfer a signal, which protects cells from apoptosis, and the alteration of this pathway leads to cell death [4, 5].

The pathways of integrin-mediated signal transduction controlling apoptosis and the roles of individual receptors in them remain obscure. In particular, it is not clear whether integrins can transmit only protective (anti-apoptotic) signal (in this case cell death from anoikis is mediated by the disruption of this signal), or these receptors are able to generate a proapoptotic signal when the cellular contacts with matrix are altered, thus leading to cell death.

The properties and functions of integrin αvβ3 have been intensively studied during recent years. This receptor possesses affinity to different macromolecules [6] and is involved in various biological processes [5, 7-9]. The role of αvβ3 in anoikis has been demonstrated in several cell types [9-13]. It was shown that the disruption of αvβ3 function by its antagonists led to apoptosis, and, on the contrary, the stimulation of the receptor by its agonists rescued cells from death. These results indicate that αvβ3 generally mediates a signal preventing apoptosis.

We demonstrate for the first time in this work that integrin αvβ3 can generate a signal stimulating apoptosis when cellular contacts with the substrate are altered.

MATERIALS AND METHODS

The study was carried out using human intestinal carcinoma cell line Caco-2. The cell line was kindly provided by Dr. A. Tayner (University of Illinois, Chicago, USA). The cells were cultivated in medium DMEM/F-12 (1 : 1) containing 16% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Rabbit polyclonal antibodies to cytoplasmic domains of α-subunits of human integrins (α1, α2, α3, α5, and α6) were kindly provided by Dr. A. Lubimov (Russian Oncology Center, Moscow). Monoclonal antibodies LM609 to human integrin αvβ3 were kindly provided by Dr. V. Koteliansky (Biogen Inc., Cambridge, USA). Monoclonal antibodies

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VNR-147 to αv-subunit of human integrin and kits for reverse transcription (RT) and polymerase chain reaction (PCR) were from Gibco/BRL (USA). Monoclonal antibodies P5D2 to β1-human subunit were obtained from the Developmental Studies Hybridoma Bank (USA). Poly(2-hydroxyethyl methacrylate) (poly-HEM) and N-hydroxysuccinimidobiotin amidocaproate (NHS-biotin) were purchased from Sigma (USA). Antisense phosphothioate oligodeoxynucleotide (ODN) 5′-gactgtccacgtctaggt-3′ that is complimentary to the sequence fragment 136-153 of mRNA of human αv-subunit, sense ODN (5′-gcacttcggcgatggctt-3′) identical to the indicated above sequence, and primers for PCR were synthesized by JSC Syntol (Moscow, Russia).

Anoikis was detected by the appearance of apoptotic signs after cultivation of the cells on a non-adhesive substrate, poly-HEM, prepared by the method described earlier [4]. Cells cannot attach to this substrate and remain in suspension. To obtain the cell population resistant to anoikis (i.e., surviving when adhesion is inhibited), the cells were cultivated for several passages on poly-HEM in the medium containing 10% serum. Afterwards the cellular suspension was placed for 2 h into Petri dishes with adhesive substrate (collagen type I) immobilized on their surface. The substrate was prepared as described earlier [14]. Only viable cells adhered to this substrate. The attached cells were suspended in phosphate buffered saline (PBS) or in culture medium and were subjected to the following analysis.

For cytofluorimetric analysis of apoptosis 10^6 cells taken before or after incubation on poly-HEM, or cells that survived after such incubation were fixed in 70% ethanol, pelleted, and suspended in 1 ml of 0.1 M citrate buffer, pH 7.5, containing 0.1% Triton X-100. Then 20 μl of propidium iodide solution (0.5 mg/ml) in PBS was added, and the suspension was incubated 1 h in the cold and analyzed on a flowing cytofluorimeter (Becton Dickinson, USA).

In the experiments on the assessment of the influence of immobilized antibodies on anoikis, apoptosis was detected by incorporation of radiolabeled thymidine in intact and degraded DNA according to the described earlier method [15] with minor modification. The initial cells in logarithmic growth phase were incubated in complete medium containing [^{14}C]thymidine (Amersham, Holland, 500 μCi/ml, 10 μCi per $1.5 \cdot 10^6$ cells) for 18 h at 37°C. Then the cells were washed with PBS for elimination of free radioactivity and incubated in complete medium for 1 h at 37°C. The cells were collected using EDTA-trypsin, suspended in the medium without serum and incubated on the substrates from immobilized antibodies (see below) at 37°C for 3.5 h. Then the cells were collected, washed, suspended in medium with 10% fetal serum, and incubated on poly-HEM for 15 h at 37°C. After incubation the cells were lysed in 20 mM Tris-HCl buffer, pH 7.4, containing 4 mM EDTA and 0.4% Triton X-100. The lysate was centrifuged at 15,000 rpm, the pellet was suspended in buffer with 1% SDS, and the radioactivities of the pellet (intact DNA) and supernatant (fragmented DNA) were measured. Apoptosis (%) was calculated as follows: radioactivity of supernatant/(radioactivity of pellet + radioactivity of supernatant).

For preparation of the substrates from immobilized antibodies, 6-well plastic plates were treated with rabbit antibodies to mouse IgG (25 μg/ml) for 4 h at 37°C and washed with PBS. Free plastic was neutralized with albumin solution (10 mg/ml in PBS, 1 h at 37°C). Then the plates were washed with medium and incubated for 18 h at 4°C with antibodies to β1, αv, αvβ3, and histocompatibility complex HLA–ABC in dilution 1 : 100. The plates were washed three times with the medium and used for cultivation of cells as described above.

For cytofluorimetric analysis of the surface expression of integrins $2 \cdot 10^5$ cells were washed with PBS and incubated for 30 min in the cold in 20-50 μl of PBS containing integrin antibodies in different dilutions. Then the cells were washed with PBS, incubated 30 min in 20-50 μl of FITC-conjugated antibodies to mouse immunoglobulins in dilution 1 : 300, washed three times with PBS, fixed with 1% formaldehyde in PBS for 1 h, and analyzed in the flow cytofluorimeter.

Biotinylation of the surface membrane proteins by NHS-biotin and integrin immunoprecipitation were carried out mainly as described earlier [16]. Initial cells and cells adhered to collagen after 48 h incubation on poly-HEM were suspended in PBS (10^7 cells/ml) and incubated with NHS-biotin (100 μg/ml, 30 min, 20°C). Under these conditions biotin binds only to the cell surface proteins. The cells were lysed in buffer containing 100 mM octyl glucoside (Calbiochem, USA), centrifuged at 15,000*g* for 20 min, and then the relative biotin content in supernatants was measured by immuno-enzymatic assay using avidin–peroxidase conjugate. Cellular extracts containing equal quantities of biotin were treated with antibodies to α-subunits of integrins (in saturating concentration), and immunoprecipitates were isolated by chromatography on the conjugate of the A protein with agarose (Sigma) and fractionated by electrophoresis in 7.5% polyacrylamide gel containing SDS under non-reducing conditions. Afterwards the proteins were transferred from the gel to nitrocellulose membrane. Immunoblots were developed with streptavidin–phosphatase conjugate (Sigma).

Total cellular RNA was isolated with guanidine isothiocyanate–phenol–chloroform using TRIzol reagent (Gibco/BRL) according to manufacturer's procedure.

Reaction with reverse transcriptase (RT) was carried out in 20 μl mixture containing 1 μg of total RNA, 1× RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol), 1.25 mM oligo(dT)$_{12\text{-}18}$, 0.5 mM dNTP, and 200 U of RT (SuperScript II,

Gibco/BRL) at 42°C for 1 h followed by incubation at 90°C for 5 min.

Polymerase chain reaction (PCR) was carried out in 25 μl mixture containing 4 μl of successive cDNA dilutions obtained in RT reaction, 1× PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM dNTP, 250 nM of sense and antisense primers, 1-2 U of *Taq* DNA-polymerase (Gibco/BRL). Glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA was used as a standard. For amplification of GAPDH cDNA primers 5′-accacagtccatgccatcac-3′ (sense, nucleotides 586-605) and 5′-tccaccaccctgttgctgta-3′ (antisense, nucleotides 1037-1018) were used. Primers 5′-gttgggagattagacagagga-3′ (sense, nucleotides 2787-2807), 5′-caaaacagccagtagcaacaa-3′ (antisense, nucleotides 3074-3054) were used for amplification of αv cDNA. β3 cDNA was amplified using primers 5′-ggggactgcctgtgtgactc-3′ (sense, nucleotides 1731-1750) and 5′-cttttcggtcgtggatggtg-3′ (antisense, nucleotides 2274-2255). The primer sequences were taken from [17] and according to GenBank and EMBL banks are unique for corresponding genes. The mixture was heated for 3 min at 94°C; amplification consisted of 30 cycles. For αv each cycle consisted of the following steps: 45 sec at 94°C, 45 sec at 56°C, 60 sec at 72°C; for β3: 1 min at 94°C, 1 min at 60°C, 2 min at 72°C; for GAPDH: 30 sec at 94°C, 30 sec at 60°C, 30 sec at 72°C. PCR products were subjected to electrophoresis in 1.5% agarose gel in Tris-borate buffer, pH 8.3, and stained with ethidium bromide.

For hybridization reaction (Northern blotting) 20 μg of total RNA was fractionated by electrophoresis in 1% agarose gel with formaldehyde and transferred to nylon membrane (Amersham). For probe preparation the band corresponding to αv-PCR product was cut from the agarose gel, and DNA was extracted using a Qiaex kit (Qiagen Inc.) and labeled with [α-^{32}P]dATP in the nick-translation reaction. Hybridization was performed at 42°C for 20 h.

To assess the influence of phosphothioate ODN on anoikis, $6 \cdot 10^4$ cells were cultivated in 48-well plates in complete medium. Corresponding ODN was added to each well in concentration 10 μM each 24 h for 72 h. Eighteen hours before the end of this time period [^{14}C]thymidine was added to the cells. The cells were incubated and treated as described above. The cells were collected with EDTA-trypsin, washed, and incubated 24 h on poly-HEM in medium containing 10% fetal serum. Then apoptosis was detected by measuring the ratio between radioactivities of fragmented and total DNA (as indicated above).

RESULTS

Expression of integrins in anoikis-positive and anoikis-negative cells. First of all it was necessary to estimate if the Caco-2 cells died on the non-adhesive substrate through the apoptotic mechanism. Nuclear DNA cleavage between nucleosomes is one of the typical signs of apoptosis. It can be visualized by electrophoresis: DNA forms a stair-like pattern that consists of a number of oligonucleotide fragments divisible by 200-300 bp [18]. The electrophoretic analysis revealed that DNA isolated from the initial culture consisted of high molecular weight aggregates, which poorly entered into agarose gel, while DNA from cells incubated on poly-HEM during 48 h was represented by both high and low molecular weight forms, which were distributed through all the gel (data not shown). On the other hand, DNA from the cells that survived after incubation in suspension during this period did not contain the typical products of DNA degradation.

The data of electrophoresis were confirmed by cytofluorimetric study after DNA was stained with propidium iodide. Apoptosis is characterized by the accumulation of cells with sub-diploid DNA content (<2*n*) [4]. Figure 1 shows that in the initial cell population apoptotic cells accounted for 4%. The extension of incubation on poly-HEM led to the increase in the content of apoptotic cells; after 48 h incubation it accounted for 54%. On the other hand, cell population that was incubated on poly-HEM for

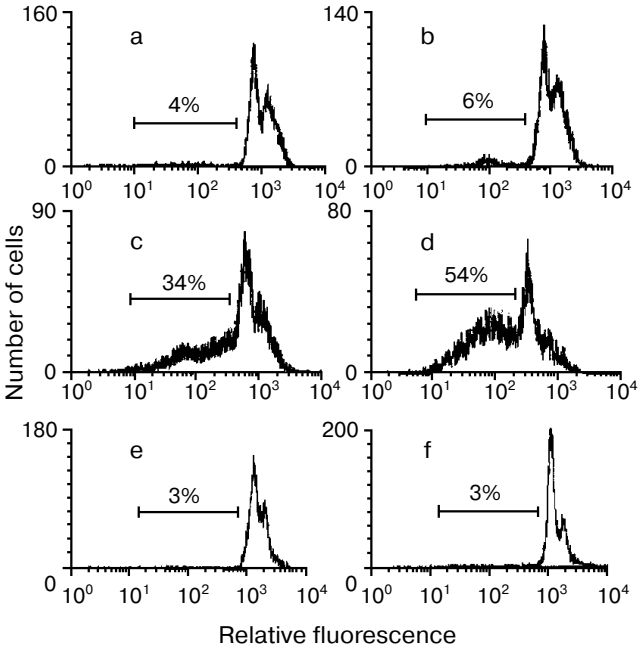

Fig. 1. Cytofluorimetric analysis of apoptosis. Cells were treated for analysis as described in "Materials and Methods": a-d) cells were incubated on poly-HEM for 0, 3, 24, and 48 h, respectively; e) cells were incubated 24 h on poly-HEM, then 2 h on collagen; the attached cells were then collected and analyzed; f) cells were treated as in (e) except that they were incubated on poly-HEM for 48 h. The relative quantity (%) of the cells with sub-diploid DNA content is indicated.

48 h and then adhered to collagen was characterized by as low content of apoptotic cells as the initial cell population.

Thus, two assays demonstrated that the substrate dependence of apoptosis was typical for initial Caco-2 cells, i.e., these cells are anoikis-positive. The cells that survived after extended incubation without adhesion to substrate (in suspension) we designated as anoikis-negative.

Interestingly, the progeny of anoikis-negative cells restored both the substrate dependence of apoptosis and the expression of αv-integrins after several passages on natural substrate. This result means that the initial cell line does not represent a stable cell population resistant to anoikis, and this resistance is not inherited by these cells.

Taking into account the role of integrins in anoikis it was interesting to estimate the differences in the surface expression of these receptors between anoikis-positive and anoikis-negative cells. The results of Western blotting demonstrated (Fig. 2) that both cell types expressed a broad spectrum of integrins. This spectrum was mainly represented by receptors that shared a common β1-subunit (subfamily β1—α1β1, α2β1, α3β1, α5β1, and α6β1). We did not find any significant difference in the expression of these receptors between the two cell types. However, anoikis-positive cells were more active than anoikis-negative cells in the expression of integrins that shared a common αv-subunit, but had different β-subunits—β1 and β3 with molecular weights 116 and 93 kD, respectively.

Cytofluorimetric analysis of the surface expression of β1- and αv-containing integrins showed that the content of the cells expressing αv decreased from 64 to 15% in the cell population, which survived after incubation in suspension for 48 h, while the content of β1-expressing cells decreased from 78 to 52% (Fig. 3). The reduction of the number of β1-expressing cells could be due to the decrease of the number of cells that express αvβ1.

To determine whether the reduction in the expression of αv-integrins in anoikis-negative cells was due to a decrease in the expression of αv-subunit gene, we performed a semi-quantitative RT-PCR assay of the content of αv-mRNA in total RNA isolated from both cell types. Figure 4a shows that the DNA fragment amplified with αv-specific primers had an expected length (288 bp) and that the anoikis-negative cells contained much smaller quantities of mRNA of αv-subunit than the anoikis positive cells. This conclusion was confirmed by hybridization of this fragment (it was used as a probe) with RNA from the analyzed cells (Fig. 4b). The probe bound to mRNA with typical for αv-subunit length (6 kb); the hybridization signal was much weaker with RNA from the anoikis-negative cells than from the anoikis-positive ones. At the same time the anoikis-positive and anoikis-negative cells did not differ in transcription of the β3-subunit gene. Thus, the reduction of the surface expression of αv-integrins in anoikis-negative cells was mediated by the inhibition of αv-gene expression.

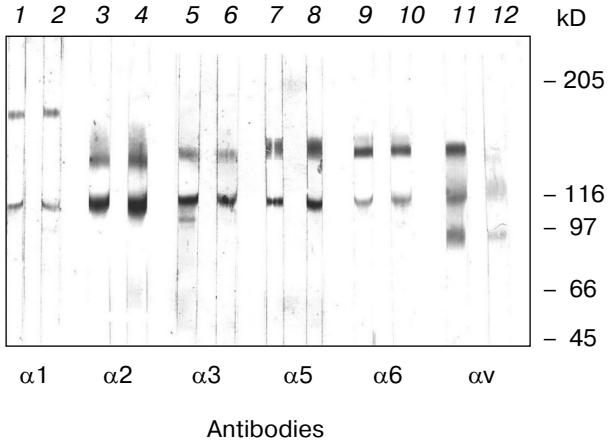

Fig. 2. Immunoprecipitation of integrins expressed on the surface cellular membrane. Biotinylation of the membrane proteins and immunoprecipitation of integrins was carried out as described in "Materials and Methods". Integrins α1β1, α2β1, α3β1, α5β1, and α6β1 were immunoprecipitated by antibodies to the cytodomain of corresponding α-subunits. αv-containing integrins were immunoprecipitated by monoclonal antibodies VNR-147 to αv-subunit. Lanes: *1*, *3*, *5*, *7*, *9*, *11*) initial cells; *2*, *4*, *6*, *8*, *10*, *12*) cells that survived in suspension. It is clear that in both cell types αv-subunit forms a dimer with β1-subunit (integrin αvβ1) with molecular weight 120 kD, and with β3-subunit (integrin αvβ3) with molecular weight 95 kD.

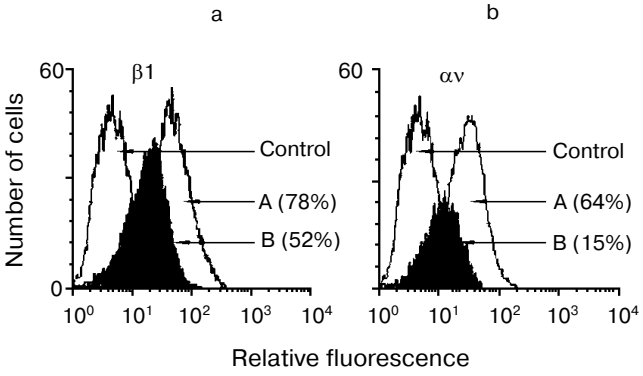

Fig. 3. Cytofluorimetric analysis of integrins expression in the cells that survived in suspension. Initial cells were incubated on poly-HEM for 0 (a) and 48 h (b). Then the surviving cells were isolated and treated with antibodies P5D2 or VNR-147 in dilutions 1 : 400 and 1 : 200, respectively, and FITC-conjugated secondary antibodies as described in "Materials and Methods". Each figure represents an assembly of three primary histograms obtained after the analysis: of the cells treated only with FITC-conjugated antibodies to mouse immunoglobulins (control); or of the cells treated first with one of indicated integrin antibodies and then with FITC-conjugated antibodies. The content of β1- and αv-expressing cells is presented in percent.

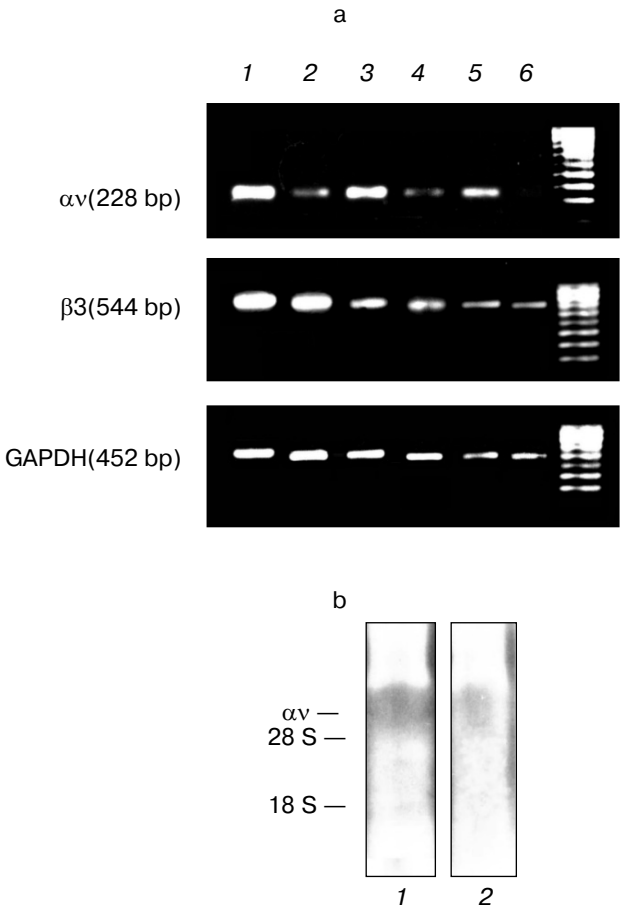

Fig. 4. RT-PCR and Northern blotting of αv-mRNA from initial cells and cells that survived on poly-HEM. a) Total RNA isolated from the initial cell culture and from the cells adhered on collagen after incubation for 48 h on poly-HEM was subjected to reverse transcription and the obtained product was added to PCR in dilutions 1 : 5 (*1*, *2*), 1 : 10 (*3*, *4*), 1 : 20 (*5*, *6*). In these dilutions the reaction was in exponential phase. Lanes: *1*, *3*, *5*) cDNA from the initial cells; *2*, *4*, *6*) cDNA from the cells surviving on poly-HEM. The sizes of PCR products in bp are indicated in brackets. αv-, β3-, and GAPDH PCR products are of expected size, the cells do not differ in the content of the control mRNA (GAPDH), but significantly differ in the content of αv-mRNA. b) Northern blotting of mRNA from the initial (*1*) and surviving on poly-HEM (*2*) cells. The positions of 28S and 18S ribosomal RNA are indicated on the left (identified by staining with ethidium bromide).

Influence of activation and inhibition of the signal activity of αvβ3 integrin on anoikis. The detected changes in αv expression in the anoikis-negative cells could be an incidental sign that was not related to the mechanism of protection from apoptosis. However, it could be suggested that in Caco-2 cells in the absence of adhesion to matrix the signal for apoptosis was mediated by αv-integrin. In this case if the initial Caco-2 population contained a certain number of cells with decreased expression of αv-integrins, these cells would be more resistant to anoikis and would survive in suspension.

To confirm this suggestion the initial cells were cultivated on immobilized antibodies to αvβ3 dimer, αv-subunit, and β1-subunit. Adhesion on such substrates leads to the formation of clusters of corresponding receptors in the membrane, their activation, and generation of a signal [19]. The cells cultured on the antibodies to the surface antigens of histocompatibility complex, HLA–ABC, were used as a control (their clusterization does not influence apoptosis) [20]. As can be seen from Table 1, activation of β1-integrins in the initial cells did not influence their further viability in the absence of the contacts with substrate. On the contrary, activation of αvβ3 integrin by both antibodies to dimer and αv-subunit considerably reduced cellular viability in suspension. This effect was observed when two methods of apoptosis detection were used.

Another approach consisted of the analysis of anoikis in the cells after the inhibition of synthesis of αv-subunit (and consequently of its surface expression) by αv-specific antisense oligonucleotide. The cells that were not treated with oligonucleotide and the cells incubated with sense oligonucleotide were taken for the control. Preliminary cytofluorimetric analysis showed that the incubation of the cells for 3 days with 10 μM of antisense oligonucleotide led to the decrease of the surface expression of αvβ3 approximately 1.5-fold (data not shown). Incubation with sense oligonucleotide had no influence on the receptor expression. Although the reduction of

Table 1. Influence of integrin antibodies used as a substrate for adhesion on anoikis of Caco-2 cells

Antibodies	Method of apoptosis detection	
	Ratio (%) of radioactivity of fragmented DNA to radioactivity of total DNA	Cytofluorimetry, % of cells with sub-diploid DNA content
anti-HLA	11.7 ± 0.7	18.5 ± 1.5
anti-β1	10.0 ± 0.6	16.5 ± 0.5
anti-αvβ3	26.3 ± 1.2	26.8 ± 2.3
anti-αv	17.7 ± 0.9	27.3 ± 1.2

Note: Radiolabeling of cells with [^{14}C]thymidine, preparation of immobilized antibodies, apoptosis detection by the ratio of radioactivities in DNA fractions and by cytofluorimetry are described in "Materials and Methods". The cells were incubated on poly-HEM for 15 h in the case of radiolabeling and for 18 h for cytofluorimetry. The results of three independent experiments with 3-4 repeated points in each are presented in the table as the mean value ± standard deviation.

Table 2. Influence of inhibition of synthesis of αv-subunit on anoikis of Caco-2 cells

Treatment of cells	Apoptosis, %
Without ODN	36.3 ± 2.1
αv-specific sense ODN	35.2 ± 2.0
αv-specific antisense ODN	23.7 ± 1.1

Note: Cells were incubated with ODN as described in "Materials and Methods". The results represent mean value ± standard deviation of three independent experiments with 4 repeated points in each.

Table 3. Apoptosis of Caco-2 cells adhered to different substrates

Substrate	Apoptosis, %
Collagen type I	4.8 ± 0.75
Anti-HLA	3.5 ± 0.64
Anti-β1	3.8 ± 0.48
Anti-αvβ3	4.3 ± 0.63

Note: Cells collected with trypsin-EDTA were washed with medium and incubated 15 h in medium with 10% fetal serum on substrates with antibodies prepared as described in "Materials and Methods". Adhered cells were detached from the substrates with trypsin-EDTA. Apoptosis was detected by cytofluorimetry after coloration with propidium iodide. The results are presented as the mean value ± standard deviation of two independent experiments with 3 points in each.

αvβ3 expression was relatively small, it had a pronounced effect on anoikis. Table 2 demonstrates that apoptosis rate in the cells cultivated for 24 h in suspension and treated with antisense oligonucleotide was 40% lower than in the untreated cells. This effect was reliable ($p < 0.01$) and specific, because treatment of the cells with sense oligonucleotide did not influence their viability.

Thus, activation of αvβ3 integrin stimulated anoikis of Caco-2 cells, and the inhibition of αvβ3 expression increased their resistance to this type of apoptosis.

If the activation of αvβ3 receptor led to the generation of the apoptotic signal in the non-adherent cells, it would be interesting to assess how cells would react on the activation of αvβ3 for cells adhered to substrate. To answer this question we were looking for apoptosis in the initial cells that were adhered to antibodies to αvβ3. The level of apoptosis in these cells did not differ from that in the cells adhered on the natural substrate collagen or on the antibodies to histocompatibility complex (Table 3). Hence, stimulation of apoptosis after activation of αvβ3 was anoikis-specific, i.e., the apoptotic signal was generated only in the case of alteration of the cellular contacts with the substrate.

DISCUSSION

In this study we demonstrated using two independent approaches that in human intestinal carcinoma cell line integrin αvβ3 can generate an anoikis-stimulating signal. It remains unknown whether this receptor performs the same activity in other cell types.

A number of studies have been devoted to the role of integrins in cellular death by apoptosis. The conclusion that in several cell types these receptors are involved in the transduction of signals that control apoptosis had been made already in early studies on the basis of the following observations: a) treatment of epithelial cells with integrin antagonist (RGD-peptide) led to their detachment from the substrate and apoptotic death [4]; b) adhesion of endotheliocytes on the immobilized antibodies to integrins (but not to other surface antigens) rescued them from apoptosis [20]; c) melanoma cells that did not synthesize αvβ3 integrin were subjected to apoptosis when cultivated in three-dimensional collagen gel, while the restoration of synthesis of this receptor by transfection of αv-cDNA prevented apoptosis [10]. These results correlate with the data that αvβ3 antagonists, which blocked αvβ3 adhesion to matrix and signal transduction, stimulated apoptosis of angiogenic cells of chicken embryos *in vivo* [11], of endothelial cells that were cultivated in three-dimensional gel [21], and also of several brain tumor cell lines [22]. On the other hand, in Chinese hamster cells and human osteosarcoma cells fibronectin-specific integrin α5β1, but not αvβ3, played an apoptosis-protecting role [23]. Thus, the involvement of individual receptors in apoptosis is determined by the cell type and the sort of apoptosis-inducing signal (for example, the absence of growth factors and disruption of adhesion to matrix).

These and other investigations devoted to the role of integrins in apoptosis gave rise to the conception that the function of these receptors generally consists of generation or transduction of a signal that protects cells from death. This conclusion was confirmed by the studies that demonstrated the influence of integrins on the activity of different phosphokinases and other intracellular proteins that play an important role in cellular viability [5, 7, 24, 25]. In particular, αvβ3 is involved in the activation of MAPK/ERK [26] and NF-κB [27], and in inhibition of p53 and $p21^{waf1}$ activities [12].

On the other hand, several recent investigations indicate that integrins may also be active in the transduction of a signal stimulating apoptosis. Thus, it was shown that transfection of carcinoma RKO cells deficient for integrin α6β4, with β4 gene followed by restoration of the expression of this receptor stimulated apoptosis [28, 29]. Proapoptotic effect of α6β4 was mediated by an activat-

ing effect of this integrin on p53. In p53-deficient cells α6β4 had an antiapoptotic effect mediated by activation of AKT/PKB phosphokinase [30]. This ambivalence was revealed after the analysis of different cell types. In certain cells (in endothelium, for example [31]) α6β4 was involved in the transduction of apoptotic signal, while in others (keratinocytes [32]) it promoted their survival.

The ability of αvβ3 integrin to mediate proapoptotic signal was demonstrated in embryonic kidney cells [13]. RGD-containing disintegrin echistatin (αvβ3 antagonist) induced apoptosis in the culture of human embryonic kidney cells before their detachment from the substrate (i.e., through anoikis), and had no effect on the cells in suspension, although it bound to αvβ3 on their surfaces. Therefore, echistatin has a double effect: after extended treatment of the culture it, as other RGD-antagonists, induces the detachment of the cells from the substrate leading to anoikis, while during shorter treatment it binds to integrin and activates signal transduction though this receptor (in this case it is an apoptotic signal), i.e., it functions as a αvβ3 agonist.

Comparing our results with the data presented above [13, 28-31], it is primarily important to state that in the cited investigations the integrin-mediated stimulation of apoptosis was studied in cells that were adhered to substrate, while we revealed the activating effect of integrin on anoikis—cell death that is induced by total disruption of cellular adhesion to substrate. It can be suggested that the mechanisms of apoptosis activation in these models differ significantly, since the cells adhered to substrate and the cells in suspension differ markedly in many morphogenetic and biochemical properties, such as cytoskeleton organization, formation of focal contacts, activity of signal-transducing mediators, etc. that have a powerful influence on cellular viability.

It was shown in recent studies [33, 34] that αvβ3 and β1-integrins can generate a proapoptotic signal in cells adhered to substrate when none of the mentioned receptors is bound to the substrate (is not ligated), i.e., when adhesion occurs on the substrate that does not contain ligands for αvβ3 or any of β1-integrins. This type of apoptosis was referred to as integrin-mediated death (IMD). It was shown that the cells of umbilical endothelium and certain tumor cells, which expressed αvβ3, died when cultivated in collagen gel (this gel is not a suitable substrate for this integrin), while cellular variants lacking αvβ3 survived under these conditions. A new mechanism of IMD was described in this work: the unligated integrin bound and activated the initiating caspase 8 and recruited it in the cellular membrane. A more detailed analysis showed that the near-membrane fragment of cytodomains of β1 and β3-integrin subunits was responsible for binding to caspase 8. The authors considered that IMD and anoikis differed in biochemical mechanisms. The data that several cell types expressing β1- and/or β3-integrins were not sensitive to IMD, but were sensitive to anoikis, favor this hypothesis [10, 23]. Caco-2 cells used in this study may also be attributed to this cell type, because the initial cell population was represented mainly by αvβ3-expressing cells and was not subjected to apoptosis when adhered to the native collagen (αvβ3-non-binding substrate) (see Table 3).

It should be stressed in conclusion that fibronectin-specific integrin α5β1 was recently found to be able to stimulate anoikis in several epithelial cell lines (when α5β1 hyperexpression was induced by hyperexpression of tumor suppressor $p16^{INK4a}$) [35]. The mechanisms of the pro-anoikis effect of α5β1 were not discussed in this paper. The discovery of the "atypical" effect of integrins on apoptosis completed in our and other studies indicates that integrins are involved in the multi-stage and effective system of the control of cell viability. This system implies that the same receptor sends a "rescuing" signal in the normal situation and switches this signal to a "mortal" one under abnormal conditions, when the death of "homeless" cells can be profitable for an organism.

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