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The $\alpha5\beta1$ integrin supports survival of cells on fibronectin and up-regulates Bcl-2 expression

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ABSTRACT **Anchorage-dependent cells that are prevented from attaching to an extracellular matrix substrate stop proliferating and may undergo apoptosis. Cell adhesion to a substrate is mediated by the integrin family of cell surface receptors, which are known to elicit intracellular signals upon cell adhesion. We show here that Chinese hamster ovary cells expressing the $\alpha5\beta1$ integrin, which is a fibronectin receptor, do not undergo apoptosis upon serum withdrawal when the cells are plated on fibronectin. However, the $\alpha v\beta1$ integrin, which is also a fibronectin receptor and binds fibronectin on the same RGD motif as $\alpha5\beta1$, did not prevent apoptosis on fibronectin of the same cells. The cytoplasmic domain of the integrin $\alpha5$ subunit was required for the $\alpha5\beta1$-mediated cell survival on fibronectin. The fibronectin-mediated survival effect appeared to be independent of the level of tyrosine phosphorylation of the focal adhesion kinase, which is induced by integrin-mediated cell attachment. The expression of the Bcl-2 protein, which counteracts apoptosis, was elevated in cells attaching to fibronectin through $\alpha5\beta1$; cells attaching through $\alpha v\beta1$ survived only if exogenous Bcl-2 was provided. Thus, $\alpha5\beta1$, but not the closely related $\alpha v\beta1$ integrin, appears to suppress apoptotic cell death through the Bcl-2 pathway.**

Most types of normal cells require attachment to extracellular matrix to be able to proliferate and differentiate, whereas this dependence on anchorage is reduced in transformed cells (1). Recent findings demonstrate that in addition to regulating cell growth and differentiation, the extracellular matrix also functions as a cell survival factor for many cell types. Failure to adhere to a substrate has been shown to activate a suicide process in endothelial and epithelial cells; if prevented from attaching, these cells undergo cell death with the morphological and biochemical characteristics of apoptosis (2–5). Cell adhesion to a substrate is mainly mediated by the integrin family of cell surface receptors (6, 7). In agreement with this, integrin-mediated events seem to be involved in the detachment-induced programed cell death. Functional blocking of integrins with RGD peptides in cultured epithelial cells detaches the cells from the substrate and induces apoptosis (3). Conversely, cell attachment to anti-$\beta1$ integrin antibodies, but not to antibodies against non-integrin cell surface molecules, prevents apoptosis (2). While these studies implicate integrins in the anchorage dependence phenomenon, they do not allow conclusions as to which particular integrin or integrins might be involved. This is one of the questions we have addressed in the present study.

A wealth of information has accumulated in the past years regarding integrin-activated intracellular signaling. Notably, several studies have shown that cell spreading and integrin clustering induce increased tyrosine phosphorylation of various intracellular proteins including the focal adhesion kinase (FAK) (8, 9). However, the integrin-mediated signals that prevent entry into a cell suicide program are not known, although integrin-stimulated tyrosine phosphorylation may be involved, because detachment-induced apoptosis of endothelial cells can be blocked by tyrosine phosphatase inhibitors (2).

Here we show, by using cell lines that express different fibronectin-binding integrins (10, 11), that cell attachment mediated by one of these receptors, $\alpha5\beta1$, can protect cells from apoptosis, whereas another fibronectin receptor, $\alpha v\beta1$, and various vitronectin receptors are ineffective in this regard. We also present evidence that the ligation of the $\alpha5\beta1$ integrin results in elevated expression of Bcl-2. This pathway appears to represent one of the mechanisms whereby certain integrin–extracellular matrix interactions can support cell survival.

MATERIALS AND METHODS

Cell Lines. The B2/$\alpha5\beta1$+ and B2/$\alpha v\beta1$+ cells were generated by introducing cDNAs coding for integrin $\alpha5$ and αv subunits, respectively, into an $\alpha5\beta1$-deficient Chinese hamster ovary (CHO) cell line (B2-CHO cells) as described (10–12). The B2/$\alpha5\Delta c\beta1$+ cells were obtained by transfecting the B2-CHO cells with a truncated $\alpha5$ construct ($\alpha5\Delta c$) that lacked the cytoplasmic domain. This construct was generated by introducing a stop codon in the human $\alpha5$ cDNA at position 3108 (13) by site-directed mutagenesis. The $\alpha5$ cDNA was then cloned into the *Hin*dIII–*Xba* I site of pRC/CMV plasmid (Invitrogen) and expressed in the B2-CHO cells as described (11). Clones expressing the $\alpha5\Delta c$ subunit were identified by fluorescence-activated cell sorting with anti-human $\alpha5$ monoclonal antibody (mAb) P1D6 (11, 14), and the identity was confirmed by immunoprecipitation with mAb P1D6 and lack of reactivity with anti-$\alpha5$ cytoplasmic domain antiserum (15). The transfectants were maintained in α minimum essential medium (α-MEM) containing 10% fetal calf serum and 250 μg of G418 per ml. Wild-type CHO-K1 and the MG-63 human osteosarcoma cells were from the American Type Culture Collection and were cultured in α-MEM with 10% fetal calf serum. Human keratinocyte cell line HaCaT was from Norbert Fusenig (German Cancer Research Center, Heidelberg).

Nuclear Fragmentation and DNA Fragmentation Assays. Subconfluent cells were detached with trypsin, washed once with serum-containing α-MEM medium, washed twice with serum-free α-MEM medium, and plated on six-well tissue culture dishes (10^6 cells per well) that had been coated with 30 μg of fibronectin per ml (Finnish Red Cross, Helsinki). At designated time points, attached and floating cells were collected by centrifugation, washed once with phosphate-buffered saline (PBS), fixed with 3.7% paraformadehyde for 10 min at room temperature, and stained with 0.1 μg of 4′,6-diamidino-2-phenylindole per ml in PBS (Sigma). After three washes with PBS, the cells were mounted onto slides for analysis under a fluorescence microscope (16). For DNA fragmentation assay,

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Abbreviations: CHO, Chinese hamster ovary; FAK, focal adhesion kinase; mAb, monoclonal antibody; RT–PCR, reverse transcription polymerase chain reaction.

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the cells were recovered after a 36-hr incubation and analyzed on 2% agarose as described (17).

Immunoprecipitation and Immunoblot Analyses. To detect tyrosine phosphorylation of FAK, cells (10^6 cells per well) plated onto six-well dishes coated with 30 μg of fibronectin per ml were incubated for 3 hr at 37°C and then lysed for 40 min on ice in a Nonidet P-40 buffer (18). The lysates were centrifuged for 20 min at 16,000 × *g* and the supernatants were immunoprecipitated with anti-FAK mAb 2A7 (Upstate Biotechnology, Lake Placid, NY). Immunoprecipitates were separated on precast 4–20% SDS/PAGE gels (NOVEX, San Diego) under reducing conditions followed by electroblotting to Immobilon-P (Millipore). Tyrosine-phosphorylated proteins were detected by immunoblotting with peroxidase-conjugated anti-phosphotyrosine mAb RC20H (Transduction Laboratories, Lexington, KY) followed by chemiluminescence detection (ECL, Amersham). To analyze the expression of Bcl-2 and Bax, cells were incubated on fibronectin-coated six-well tissue culture dishes for 48 hr. Cells were detached with trypsin and lysed in RIPA buffer (11) for 30 min on ice. After a 20-min centrifugation at 16,000 × *g*, protein concentrations of the supernatants were measured by Bio-Rad protein assay kit. Lysates containing equal amounts of proteins were stained with polyclonal anti-Bcl-2 and anti-Bax antisera (19, 20) followed by chemiluminescence detection (ECL) with peroxidase-conjugated protein A (Sigma).

Detection of *bcl-2* by Reverse Transcription Polymerase Chain Reaction (RT-PCR). Poly(A)$^+$ RNAs were isolated from the cells incubated on fibronectin for 48 hr by Micro FastTrack mRNA isolation kits (Invitrogen). A semiquantitative RT-PCR was employed to detect the relative levels of *bcl-2* and β_2-microglobulin mRNAs as described (19). The forward primers (*bcl-2*, 5′-TGCACCTGAGCGCCTTCAC-3′; β_2-microglobulin, 5′-ATGGCTCGCTCGGTGACCCTAG-3′) and reverse primers (*bcl-2*, 5′-TAGCTGATTCGACCATTTGCCTGA-3′; β_2-microglobulin, 5′-TCATGATGCTTGATCACATGTCTCG-3′) were chosen according to mouse *bcl-2* and β_2-microglobulin sequences. Amplification was done at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min. Each cDNA was amplified for 20 cycles.

Retroviral Expression of *bcl-2*. The B2/α5Δcβ1+ and B2/αvβ1+ cells were infected with retrovirus pLXSHD-bcl-2 (B2/α5Δcβ1+/bcl-2 and B2/αvβ1+/bcl-2), which contains a *bcl-2* cDNA and a histidinol selection gene (gift of Fred Gage, University of California, San Diego). The same parental cells infected with the retrovirus pLXSHD were used as controls (B2/α5Δcβ1+/c and B2/αvβ1+/c). Prior to infection, cells were treated with 0.18 μg of tunicamycin per ml for 20 hr, and infections were done for 5 hr in medium containing 10 μg of Polybrene per ml. Cell clones stably expressing *bcl-2* were selected with 2.5 mM of L-histidinol (Sigma). The expression of *bcl-2* was detected by immunoblotting with anti-human Bcl-2 antiserum (19, 20).

RESULTS

Cell Attachment Through α5β1 Integrin Promotes Cell Survival. We have previously demonstrated that the introduction of the α5 integrin subunit by cDNA transfection into an α5β1-deficient B2-CHO cell line (10) results in the expression of the α5β1 integrin (B2/α5β1+ cells), whereas transfection with the αv subunit cDNA yields expressors of the αvβ1 integrin (ref. 11; B2/αvβ1+ cells). These cell lines express the transfected integrins at similar levels and attach and spread on fibronectin to the same extent (10, 11). A cell line generated by transfecting the B2 cells with an α5 construct lacking the cytoplasmic domain (B2/α5Δcβ1+ cells) adhered to fibronectin equally well while spreading less well than cells transfected with full-length α5 or αv (data not shown; ref. 21).

Despite their similar attachment to and spreading on fibronectin, the B2/α5β1+ and B2/αvβ1+ cells exhibited different survival on fibronectin-coated dishes in the absence of serum. After 4 days following serum withdrawal, nearly 100% of two independently derived B2/αvβ1+ cell lines displayed morphological evidence of apoptotic cell death, such as cell shrinkage and membrane blebbing (Fig. 1). Moreover, as shown in Fig. 1 *B* and *C*, nuclear fragmentation and oligonucleosomal-length DNA fragmentation had occurred in these cells. In contrast, <25% of the B2/α5β1+ cells had become apoptotic during the same time period. However, the B2/α5Δcβ1+ cells that express the α5 subunit lacking the cytoplasmic domain were highly susceptible to apoptosis (Fig. 1). All three cell types exhibited nuclear fragmentation upon serum withdrawal when plated onto poly(lysine)-coated dishes or onto uncoated cell culture dishes (data not shown). These results suggest that the fibronectin–α5β1 integrin interaction protects cells from apoptosis, whereas the fibronectin–αvβ1 integrin interaction does not. Moreover, the cytoplasmic domain of α5 appears to be needed for the protection against apoptosis by α5.

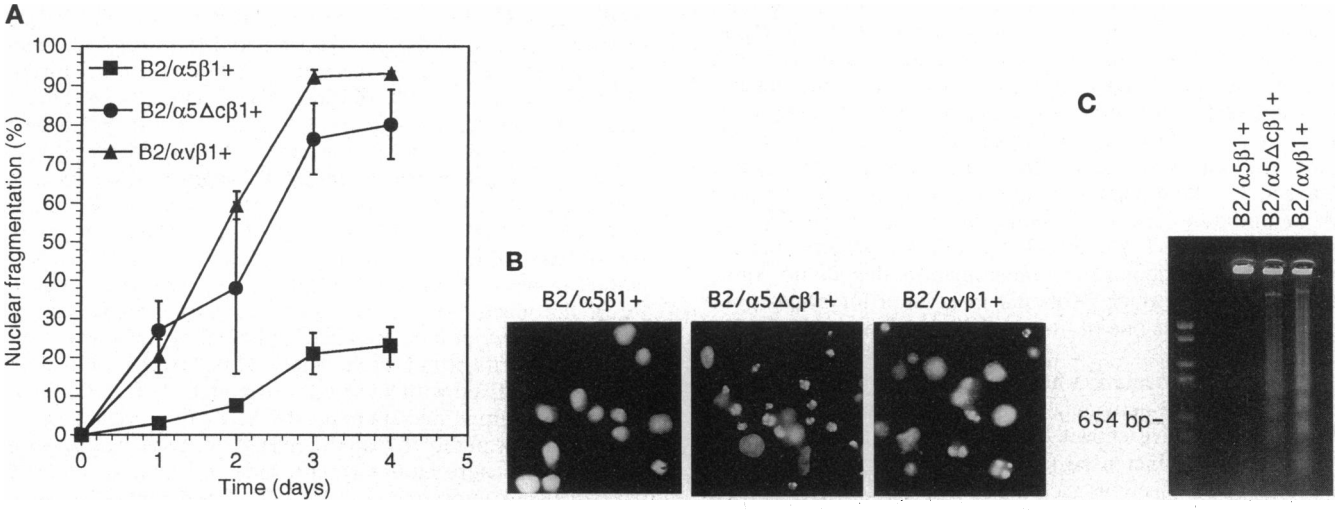

FIG. 1. Nuclear fragmentation in CHO cell transfectants plated on fibronectin. (*A*) Quantitation of nuclear fragmentation in B2-CHO transfectants. The cells were incubated on fibronectin-coated (30 μg/ml) dishes in serum-free medium. At indicated time points, the cells were stained with 4′,6-diamidino-2-phenylindole and nuclear fragmentation was scored using a Nikon fluorescence microscope. (×40.) Standard deviations of the means are shown. (*B*) Analysis of nuclear fragmentation. The cells were processed for microscopy as in *A* after 36 hr in culture. (*C*) DNA fragmentation. Cells were collected after a 36-hr incubation and analyzed by agarose gel electrophoresis.

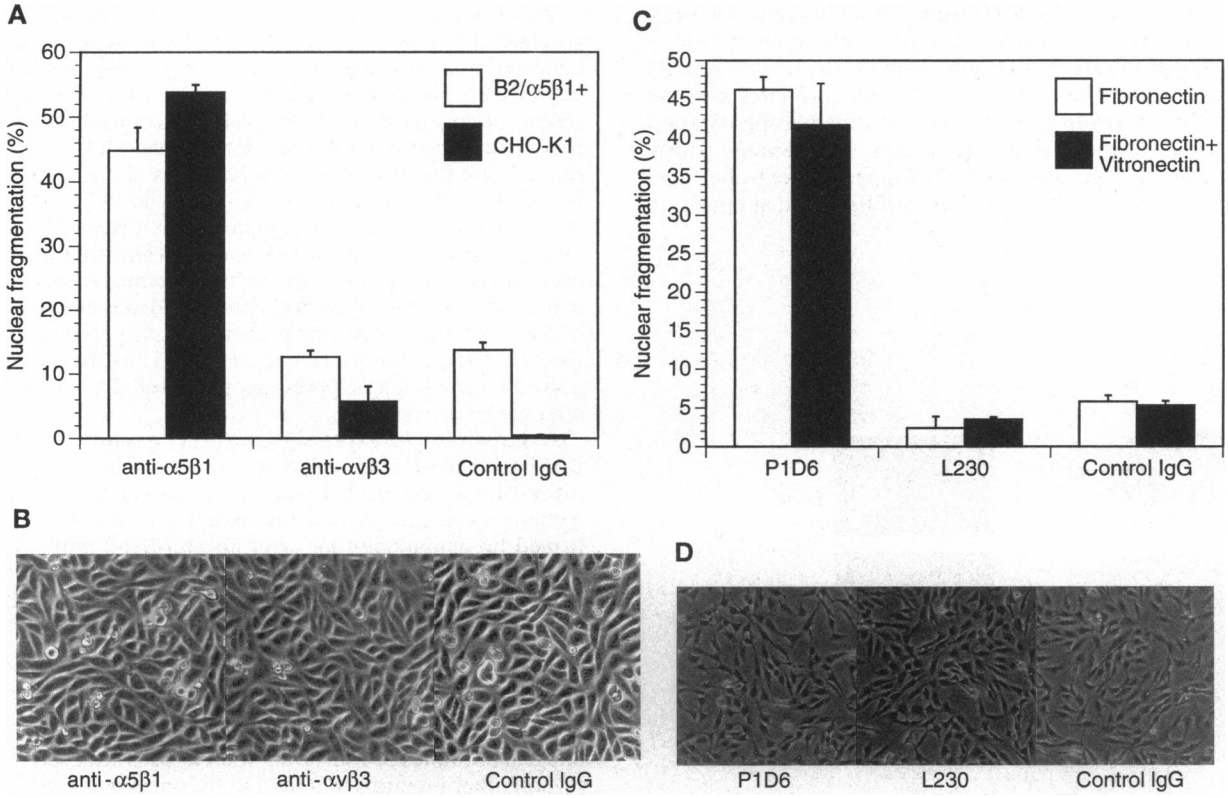

FIG. 2. Effect of anti-integrin antibody treatment on nuclear fragmentation. (*A*) Nuclear fragmentation of B2/α5β1+ and CHO-K1 cells. Cells were plated on six-well tissue culture plates coated with a mixture of fibronectin (15 μg/ml) and vitronectin (15 μg/ml) in the presence of the following antibodies: affinity-purified rabbit anti-α5β1 IgG (150 μg/ml, "anti-α5β1" for B2/α5β1+), mAb PB1 (100 μg/ml, "anti-α5β1" for CHO-K1), affinity-purified rabbit anti-αvβ3 IgG (150 μg/ml, "anti-αvβ3"), and normal rabbit IgG (150 μg/ml, "control IgG"). (*B*) Attachment of CHO cells to a mixture of fibronectin and vitronectin. Cells were incubated on dishes coated with a mixture of fibronectin and vitronectin in the presence of the indicated antibodies, as in Fig. 1*A*. After 12 hr, cells were photographed. (*C*) Nuclear fragmentation of MG-63 cells. Cells were plated on fibronectin (30 μg/ml) or on a mixture of fibronectin (15 μg/ml) and vitronectin (15 μg/ml) in the presence of an anti-α5 mAb (P1D6, 150 μg/ml), an anti-αv mAb (L230, 150 μg/ml; ref. 22), or normal mouse IgG (150 μg/ml, "control IgG"), as indicated. Nuclear fragmentation was assayed after a 48-hr incubation at 37°C, as in Fig. 1*A*. (*D*) Attachment of the MG-63 cells to a mixture of fibronectin and vitronectin. The experiment was carried out as described in *B* in the presence of the indicated antibodies. In *A* and *C*, results are representative experiments independently conducted three times; standard deviations of the means are shown.

The integrin specificity of cell survival on fibronectin was further confirmed by an antibody inhibition assay. To inhibit individual integrins without causing cell detachment, the test cells were plated onto a mixed substrate containing fibronectin and vitronectin in equal quantities. The parental CHO cells and the B2 variants express the αvβ5 integrin, which mediates their attachment to vitronectin (10, 11). The B2/α5β1+ cells remained attached on the fibronectin/vitronectin mixed surface even in the presence of an anti-human α5β1 polyclonal antibody and anti-α5 mAb (not shown), each of which blocks α5β1-mediated cell attachment to fibronectin but not to vitronectin, as well as in the presence of an anti-human αvβ3 polyclonal antibody that blocks attachment to vitronectin but not to fibronectin (results not shown; refs. 10 and 11). Blocking the α5β1 integrin with an anti-α5β1 polyclonal antibody induced an apoptotic response (Fig. 2*A*), even though cell attachment was not detectably reduced owing to the fact that vitronectin attachment was preserved. In contrast, no significant effect on apoptosis was seen when vitronectin-mediated attachment was blocked with an anti-αvβ3 polyclonal antibody. Normal mouse or rabbit IgG also had no effect. The CHO-K1 cells, which express endogenous α5β1, as well as αvβ5, gave results similar to those described above for the B2/α5β1+ cells (Fig. 2*A*). The attachment of these cells to fibronectin was blocked with the anti-CHO cell α5β1 mAb, PB1 (23). Similarly to the B2/α5β1+ cells, CHO cell attachment to the mixture of fibronectin and vitronectin was not reduced in the presence of either anti-α5β1 or anti-αvβ3 antibody (Fig. 2*B*). The effect of α5β1 on apoptosis was not limited to the CHO cells; the human osteosarcoma cell line MG-63 (Fig. 2 *C* and *D*) and human keratinocyte cell line HaCaT (not shown) displayed a similar response.

Binding of Cells to Fibronectin Through the α5β1 Integrin Enhances Bcl-2 Expression. Having established the role of the α5β1–fibronectin interaction in supporting cell survival, we examined the biochemical signals that mediate the cell survival. One candidate signaling molecule is FAK. FAK becomes tyrosine phosphorylated and activated upon integrin ligation, which suggests its involvement in some aspect of integrin-mediated signal transduction (8, 9). We found, however, no

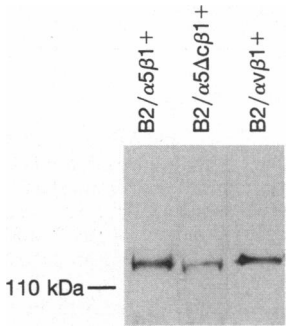

FIG. 3. Tyrosine phosphorylation of FAK. Detection of tyrosine phosphorylation of FAK was carried out as described in the text.

differences in the level of tyrosine phosphorylation of FAK between the B2/α5β1+ and B2/αvβ1+ cells upon cell adhesion on fibronectin (Fig. 3). In agreement with previous reports that the tyrosine phosphorylation of FAK correlates with the extent of cell spreading, the level of tyrosine phosphorylation of FAK was lower in B2/α5Δcβ1+ cells, which spread poorly on fibronectin. Thus, no correlation appears to exist between the level of tyrosine phosphorylation of FAK and apoptosis in the CHO cells.

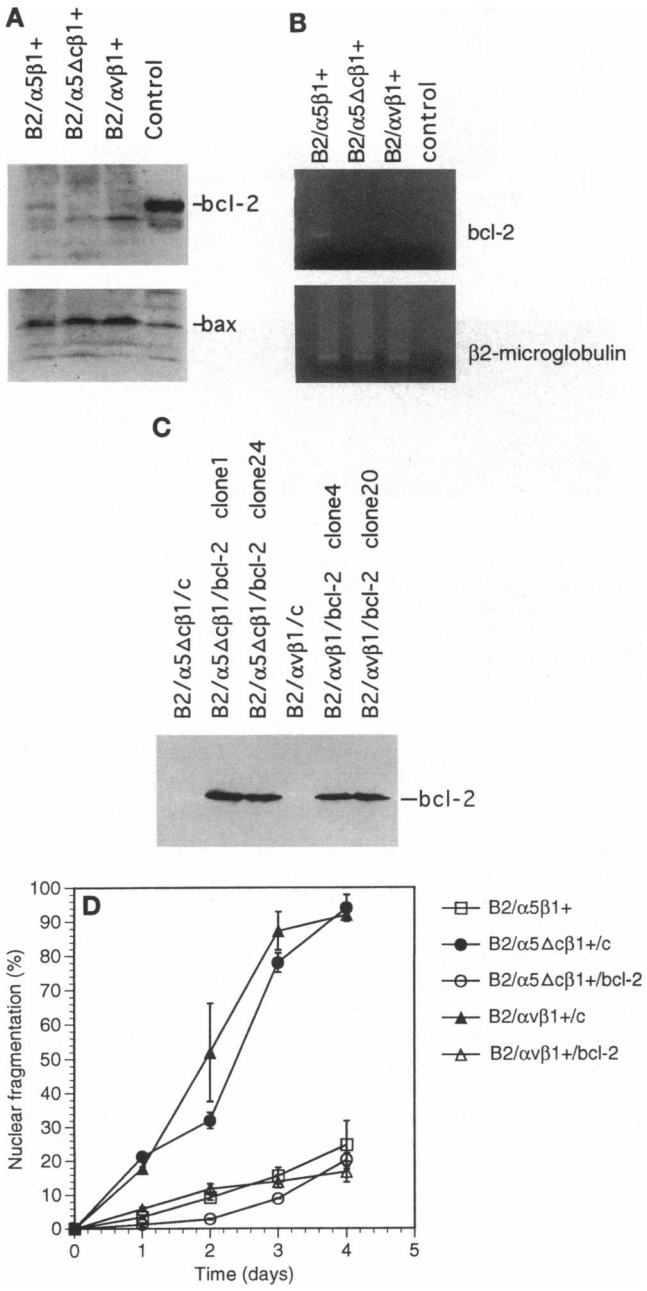

FIG. 4. Expression of *bcl-2* and *bax* in B2-CHO transfectants and prevention of apoptosis by *bcl-2* transfection. (*A*) Immunoblot analysis of *bcl-2* and *bax* expression in B2-CHO transfectants. Cell lysate from 32D cells transfected with human Bcl-2 (25) was used as a positive control ("control"). (*B*) Analysis of *bcl-2* mRNA expression by RT-PCR. Samples without adding template DNA into the PCR were used as negative controls ("control"). (*C*) Detection of *bcl-2* by immunoblotting B2-CHO cells infected with a retroviral *bcl-2* cDNA expression vector. (*D*) Nuclear fragmentation of *bcl-2*-expressing B2-CHO cells. The data for each point were generated by using a mixture of two independent cell clones plated on fibronectin. Standard deviations of the means are shown.

We next asked whether fibronectin–α5β1 interaction could regulate the expression of *bcl-2* and *bax* genes, which are believed to be two important regulators of apoptosis (24). The B2/α5β1+ cells, when plated onto fibronectin in the absence of serum, up-regulated *bcl-2* (Fig. 4*A*). In contrast, *bax* was expressed at similar levels in each of the three cell lines. RT-PCR showed that the mRNA level of *bcl-2* was also elevated in the B2/α5β1+ cells compared to the other cell lines (Fig. 4*B*). When serum was present and no apoptosis was detected, *bcl-2* was expressed at similar levels in each of the cell lines (not shown). No expression of interleukin 1β-converting enzyme, which is another important protein involved in apoptosis (26), was detected by any of these cell lines employing primers designed according to the mouse cDNA sequence (data not shown). Thus, the fibronectin–α5β1 interaction may suppress apoptotic cell death by inducing *bcl-2* gene expression.

A retroviral *bcl-2* expression construct was used to infect the B2/αvβ1+ and B2/α5Δcβ1+ cells to determine whether *bcl-2* expression would block apoptosis in these cells. Stable transfectants were cloned, and the expression of *bcl-2* was confirmed by immunoblotting with an anti-Bcl-2 antibody (Fig. 4*C*). Two independent *bcl-2*-expressing clones from each transfection were tested and shown to survive on fibronectin; cells infected with vector alone underwent apoptosis (Fig. 4*D*).

DISCUSSION

We show here that cell attachment mediated by the α5β1 integrin promotes cell survival, whereas cells attached through several other integrins that bind to fibronectin and vitronectin undergo apoptosis. We also show that the α5β1 effect is likely to be mediated by Bcl-2.

The cell survival-promoting effect of the α5β1 integrin is somewhat surprising given earlier observations that have identified this integrin as a suppressor of cell growth and tumorigenicity (27–29). However, there is precedence for such a situation; the low-affinity nerve growth factor receptor p75 promotes apoptosis when the growth factor is not present but has a survival effect when it is present (30).

Inhibition of the αvβ3 integrin can cause apoptosis of melanoma cells cultured in collagen gels and of endothelial cells engaged in angiogenesis *in vitro* (31, 32). These findings would appear to be contradictory to ours, as we found that the survival-promoting effect was specific for α5β1 among the integrins we studied. However, it is not clear whether the lack of survival in the αvβ3 studies was precipitated by lack of cell adhesion, which causes apoptosis (2, 3), or whether it might have resulted from αvβ3 inhibition similar to what we observe with α5β1.

The α5β1 integrin promotes fibronectin matrix formation (11, 33). However, the survival effect did not correlate with the presence of a fibronectin matrix. We studied two cell lines that produce matrix, B2/α5β1+ (11) and B2/α5Δcβ1+ (ref. 33; unpublished results); the former survived, but the latter did not.

The correlation we found between Bcl-2 expression and cell survival on fibronectin suggests that an elevated Bcl-2 level is the underlying factor in the survival effect by α5β1. Because the levels of Bax, which promotes cell death, were similar in the cell lines that survived and those that died, it may be that Bcl-2 protected the cells by exerting its neutralizing effect on Bax (24, 34–36).

Apoptosis of cells attached to a substrate through integrins other than α5β1 was observed only in the absence of serum; if serum was present these cells survived as well as the α5β1-expressing cells (data not shown). One view of apoptosis is that it is a cellular response to conflicting signals (37). In the absence of the growth factors contained in serum, a growth signal from an integrin could create a conflicting signal situation and drive the cell into apoptosis, it may be that attachment through the α5β1 integrin generates a signal that

parallels rather than conflicts with serum withdrawal, whereas other integrins could perhaps provide a growth-promoting signal; our recent finding that the $\alpha v\beta 3$ integrin cooperates with growth factors (18) provides some support to this notion. As serum growth factors are not normally present in tissues, serum-free culture conditions may approximate tissue environment. That cells attached through certain integrins can undergo apoptosis may be of a physiological significance in ensuring the elimination of cells that have lodged in an inappropriate tissue localization. It will be important to elucidate the molecular mechanisms of these integrin-specific signals.

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