Vitronectin, a Glioma-derived Extracellular Matrix Protein, Protects Tumor Cells from Apoptotic Death[1]

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ABSTRACT

Vitronectin (VN) is an extracellular matrix (ECM) protein, the synthesis of which *in vivo* by glioma cells correlates with tumor grade. Although the role of VN as a permissive substrate for glioma migration has been well characterized, its role in conferring a survival advantage for tumor cells has not been addressed previously. By using an *in vitro* assay of DNA fragmentation as a quantitative measure of apoptotic cell death, we sought to determine whether the sensitivity of two human glioma cell lines (D54 and U251) to drug-induced apoptosis could be inhibited by VN. As well, the extent to which apoptosis could be inhibited was correlated with the levels of the Bcl-2 family of proteins that are known to modulate apoptosis and chemoresistance. Results of the study were: (*a*) VN coatings, in a dose-dependent manner, inhibited topoisomerase (Topo)-induced apoptosis by up to 50% (optimal coating density, 500 ng/cm^2); in contrast, fibronectin (FN), an ECM protein present in abundance in the brain, demonstrated no protection; (*b*) in a dose-response study, VN clearly conferred a survival advantage (LD_{50} of Topo: on VN, 120 ng/ml; on FN, 35 ng/ml); (*c*) the protective effect of VN was not due to enhanced cell adhesion or alterations in the cell cycle distribution; (*d*) both of the classic integrin receptors that bind VN ($\alpha_v\beta_3$, $\alpha_v\beta_5$) were capable of mediating this protective effect, because ligation of either of the two classic integrins conferred chemoresistance to Topo; and (*e*) chemoresistance observed with VN was associated with an increase in expression of two antiapoptotic proteins, Bcl-2 and Bcl-X_L, with a consequent increase in the ratios for Bcl-2:Bax and Bcl-X_L:Bax. VN, an ECM protein preferentially expressed at the tumor-brain interface *in vivo*, may confer a survival advantage to glioma cells at the advancing tumor margin and may thus, in part, underlie the high level of tumor recurrence at this interface.

INTRODUCTION

Despite the many therapeutic strategies used in the treatment of malignant gliomas, the prognosis for patients afflicted with this cerebral malignancy remains poor. One of the critical factors that underlies this outcome is the almost invariable resistance of high-grade gliomas to conventional chemotherapeutic agents. Specifically, the chemoresistant phenotype may be a distinct property of tumor cells residing at the periphery of the tumor bed, because >90% of tumor recurrences in glioblastoma patients occur at the tumor margins (1). These observations have led to our postulate that biochemical conditions unique to the region of the advancing glioma margin may confer a survival advantage to tumor cells. In this regard, we have reported previously that the expression of a glioma-derived ECM[3] protein, VN, is expressed predominantly at the tumor margin (2). In the present study, we addressed the hypothesis that this ECM protein may contribute to the chemoresistant phenotype.

In the central nervous system, VN expression is abundant in the developing retina, where it functions as a positive modulator of neurite outgrowth (3) and in the developing brain (4). Although the normal adult cortex and white matter are devoid of VN, under certain pathological conditions, VN is reexpressed in the brain parenchyma as well as in the endothelial cell basement membrane (2). In this regard, we have reported previously that glioma cells express VN and its cognate receptors (the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins) at the tumor-brain interface (2). Moreover, this expression correlates with tumor grade, being most abundant in gliomas of high-grade histology (glioblastoma), less in intermediate-grade gliomas, and virtually absent in low-grade lesions (2, 5). With respect to its putative role at the advancing glioma margin, numerous studies have shown that VN and its receptors enhance glioma motility and invasiveness (5, 6).

In this report, we addressed the novel hypothesis that a potential alternate role for VN is to confer a survival advantage for glioma cells. This postulate is based on several recent observations that suggest that the interaction between a cell and its surrounding ECM serves not only as a substrate for adhesion and motility, but that this interaction also constitutes a signal

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[3] The abbreviations used are: ECM, extracellular matrix; VN, vitronectin; Topo, topoisomerase; TUNEL, terminal deoxynucleotidyl dUTP nick end labeling; FN, fibronectin; PI, propidium iodide; FACS, fluorescence-activated cell sorter; cycRGD, cyclic RGD.

that inhibits apoptotic cell death (7). With respect to VN, its role as a cell survival factor has been illustrated in endothelial cells, in which ligation of the VN receptor ($\alpha_V\beta_3$ integrin) dramatically inhibits apoptotic death, whereas antagonism of this receptor is lethal (8). In the present study, we used an *in vitro* model in which glioma cells were treated with the Topo I inhibitor, topotecan, an agent known to cause apoptotic death of these cells, to determine what effect the presence of VN would have on drug-induced programmed cell death as well as on the expression of the Bcl-2 family of antiapoptotic proteins.

MATERIALS AND METHODS

Cell Culture. Glioma cells were regularly passaged with buffered 0.05% trypsin/0.5 mM EDTA and maintained in plastic tissue culture flask (Falcon, Bedford, MA) with F-12 medium supplemented with antibiotics (penicillin/streptomycin); incubators were set at 37°C with 5% CO_2. Although cell cultures were maintained in medium containing 10% fetal bovine serum, all experiments were performed under serum-free conditions, because serum contains several ECM proteins, including FN and VN. Serum-free medium was supplemented with ITS (insulin, transferrin, selenium; Life Technologies, Inc., Rockville, MD), a standard additive for cells grown in serum-free conditions.

Coating with ECM and Anti-Integrin Antibodies. Tissue culture plastic surfaces were coated with either human FN or VN (Life Technologies, Inc.) to a surface concentration of 0–500 ng/cm^2 with modifications of methods reported previously (9). FN was chosen as a control ECM protein, because it is recognized as a ligand by a different integrin receptor. Both VN and FN localize to the focal adhesion sites in glioma cells *in vitro* and are equipotent as substrates for glioma cell adhesion and migration (6). Moreover, both FN and VN receptors have been shown to inhibit apoptosis of endothelial cells (7, 8). An important difference, however, is that VN is expressed to a greater extent by glioma cells *in vivo* (2, 5) and as such may mediate a function that is distinct from FN, such as chemoresistance. Topotecan, a Topo I inhibitor, was chosen as a chemotherapeutic stressor, because it is known to cause programmed cell death in glioma cells and the doses of Topo necessary to achieve apoptotic cell death have been well established (10). Thus, whether VN was capable of altering the sensitivity to topotecan-induced apoptosis constituted the primary area of focus in this study. In this report, we compared the effect of plating glioblastoma cell lines on VN or FN in the presence of a chemotherapeutic agent and determined the extent of apoptosis with an *in vitro* assay of DNA fragmentation.

For apoptosis experiments, 100 μl of ECM solutions diluted in PBS were aliquoted into the chambers (LabTek/NUNC, Rochester, NY), left at room temperature for 2 h, and incubated with 0.1% BSA in PBS for 30 min at 37°C to block noncoated areas of plastic with interspersed PBS washes. Chamber slides were then air-dried and used immediately. For immunoblot- and cell surface-labeling experiments, 100-mm dishes (Falcon) were coated with FN or VN (Life Technologies, Inc., Rockville, MD) at 500 ng/cm^2 by incubating the plates with 8 ml of the ECM proteins diluted in PBS. BSA blocking and washing steps, identical to those used for chamber slide coatings (described above), were then used.

Chamber slides were coated with antibodies capable of ligating specific VN receptors by methods described elsewhere (11). In brief, the slides were pretreated with goat anti-mouse IgG (100 μl of 50 μg/ml solutions) for 1 h, followed by incubation with 1% BSA for 30 min. Onto slides coated with anti-mouse IgG, the following mouse-derived purified monoclonal antibodies were added (100 μl of 20 μg/ml solutions; 1 h at room temperature): (*a*) control mouse IgG; (*b*) anti-$\alpha_5\beta_1$ integrin (ligates FN receptor); (*c*) anti-$\alpha_v\beta_3$ integrin (ligates VN receptor; this antibody is the LM609 clone); or (*d*) anti-$\alpha_v\beta_5$ integrin ($\alpha_v\beta_5$ is also a VN-binding receptor). All antibodies were purchased from Chemicon (Temecula, CA). All incubations were performed at room temperature; between each step, the chambers were rinsed three times with PBS. The chambers were allowed to air dry and used immediately for cell seeding.

Apoptosis Assay. Cells were seeded onto ECM or antibody-coated chamber slides by the following method: (*a*) flasks of cells grown in the presence of serum were rinsed twice with PBS and harvested in suspension with buffered 2 mM EDTA (trypsin was not used to avoid proteolytic cleavage of cell surface receptors for VN or FN); (*b*) cells were resuspended in serum-free F-12 medium supplemented with ITS (see above), and 1.5×10^4 cells in 200 μl were seeded to each chamber; (*c*) after 12 h, cells were treated with topotecan, and 48 h later, they were assayed for apoptosis with an *in situ* method for detecting DNA fragmentation (TUNEL) by methods described previously (12).

In brief, the TUNEL assay was performed by first washing cells in chamber slides with PBS and then fixing them in acetone:methanol (1:1 mixture) for 20 min at −20°C. Cells were then rehydrated for 30 min at room temperature. Each coverslip was then incubated in a humidified chamber at 37°C for 30 min in the following: 0.5 μl of TdT enzyme (Promega Corp., Madison, WI), 10 μl of 5× TdT reaction buffer, 1 μl of biotin-dUTP (Boehringer Mannheim, Indianapolis, IN), and double-distilled H_2O to make up 50 μl total volume. Coverslips were then washed and subsequently incubated in streptavidin conjugated to FITC (1:100 dilution) for another 30 min at 37°C. Cells were washed again in PBS and then incubated for 10 min at room temperature in 10 μg/ml PI, which counterstains all nuclei. Coverslips were mounted using glycerol/PBS and visualized by fluorescence microscopy. Apoptotic cells were defined as those cells whose DNA was labeled by TUNEL and that demonstrated other morphological characteristics of apoptosis, including nuclear blebbing and eccentrically displaced and condensed nuclei. To quantitate the percentage of cells demonstrating DNA fragmentation, the number of TUNEL-labeled cells were divided by the number cells counterstained with PI present in five random microscopic fields.

Treatment with RGD Peptides. In these experiments, topotecan was not added to the glioma cell cultures; rather, the ability of VN receptor antagonists to independently cause tumor cell apoptosis was assessed. To determine the effect of VN receptor antagonism on cell survival, glioma cells were treated with one of three peptides (Life Technologies, Inc.) used at a concentration of 100 μM: (*a*) cycRGD [G-Pen-G*RGD*SPC (RGD tri-amino acid sequence is italicized)], a peptide that has been shown to selectively antagonize the VN receptor [the VN receptor-inhibitory concentration of 100 μM is close to an order

of magnitude below the threshold for inhibition of the FN receptor (13)]; (*b*) G*RGD*SP, which typically can inhibit all RGD-mediated integrin interactions (including FN or VN receptor) but is significantly less effective than cycRGD in inhibiting the VN receptor (6, 13); or (*c*) G*RGE*SP, a control peptide that does not interfere with integrins. Cells were suspended medium containing 10% fetal bovine serum, which is a rich source of VN. While in suspension, the cells were treated with peptides for 5 min, following which the mixtures were seeded onto coverslips precoated with FN (500 ng/cm^2). By this approach, we were able to control for cell adhesion, because cells were seeded onto chamber slides in conditions that would permit adhesion/spreading via either VN, or alternately, FN, a ligand that is as equally adhesive as VN for glioma cells (6). After 48 h, cells were assayed by TUNEL to determine whether peptide treatment influenced susceptibility to apoptosis. To confirm that cell adhesion was not adversely affected in the cycRGD-treated cells, adhesion assays were performed as described previously (6).

Immunolabeling of Cell Surface Receptors for VN. All immunolabeling steps were performed with live, nonfixed cells to selectively label cell surface antigens. Glioma cells, plated on 100-mm dishes ($\sim 1 \times 10^6$ cells/dish) coated previously with 500 ng/cm^2 of FN or VN (serum-free condition; cells were plated for duration of 48 h), were rinsed with PBS and then harvested into suspension with buffered 2 mM EDTA without trypsin. After centrifugation at 1000 rpm in a Sorvall desktop centrifuge at 4°C, the cell pellet was gently resuspended in 1 ml of PBS, followed by addition of either control (mouse IgG) antibody or primary monoclonal antibodies (final concentration, 1 μg/ml) that specifically recognize the native human receptors for VN ($\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins) or FN ($\alpha_5\beta_1$ integrin) in their heterodimeric form (Chemicon). The cells were washed three times with PBS, resuspended in 1 ml of PBS, and then incubated with biotinylated anti-mouse IgG. After three washes, streptavidin-FITC (1:150) was added to the cells. After another three washes, the cell pellet was resuspended in 2% paraformaldehyde and analyzed on an EPICS FACS. All steps were performed using Ca^{2+}/Mg^{2+}-free PBS.

Immunoblotting. Chemiluminescent immunoblot analysis for the expression of Bcl-2, Bcl-X_L, and Bax was performed as described previously (14). Glioma cells were plated onto 100-mm dishes coated with either FN or VN (500 ng/cm^2) and grown in serum-free conditions as described above (with or without topotecan; see Fig. 4). After 48 h, the cells were washed with PBS and then lysed with ice-cold triple detergent buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP40, and 0.5% sodium deoxycholate]. After incubation on ice for 1 h, the lysate was centrifuged at 10,000 × *g*, and the resultant supernatant was analyzed for protein by the BCA assay (Pierce, Rockford, IL) and then mixed with 4× SDS sample buffer. Forty μg of protein were electrophoresed in 15% SDS-polyacrylamide gels, transferred overnight to Immobilon membranes, blocked with 5% milk/PBS, and then incubated with primary monoclonal antibodies diluted in 5% skim milk/PBS for 2 h at room temperature. Primary antibodies consisted of anti-Bcl-2 [mouse anti-human Bcl-2, 1 μg/ml (Oncogene Research, Cambridge, MA)], anti-Bcl-X_L [rabbit anti-Bcl-$X_{L/S}$ (Santa Cruz; Santa Cruz, CA), which recognizes both Bcl-X_L (M_r 29,000) or Bcl-X_S (M_r 19,000)] and anti-Bax [rabbit anti-human Bax, 0.3 58 g/ml (Santa Cruz; Santa Cruz, CA)]. Secondary antibodies (goat-derived) conjugated to horseradish peroxidase (Amersham, Piscataway, NJ) were used at 1:1000 dilution (1 h at room temperature), after which the membranes were exposed to horseradish peroxidase substrate (Blaze substrate; Pierce) for 5 min. Chemiluminescent signals were developed with Hyperfilm and quantitated using a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA).

RESULTS

VN Confers Resistance to Drug-induced Apoptosis. In the first series of experiments, glioma cells were plated on increasing concentrations of ECM coatings and subsequently treated with 20 ng/ml of topotecan, a dose that has been shown to be attainable in the cerebrospinal fluid (15) and associated with induction of apoptosis in both U251 and D54 glioma cells (10). An ECM coating concentration of 500 ng/cm^2 was demonstrated to be optimal in dose-response studies; VN concentrations above this level (*i.e.*, 750-1000 ng/cm^2) were associated with diminished cell survival (data not shown).

As illustrated in the micrographs of Fig. 1, the number of U251 cells that were positively labeled with the TUNEL technique in the presence of topotecan was significantly reduced in the population seeded on VN as compared with those seeded on FN at coatings of 500 ng/cm^2. For both U251 and D54 cells, the antiapoptotic effect of VN was dose dependent, with an inhibition of apoptosis of up to 50% on a 500 ng/cm^2 coating of VN, whereas in contrast, cells plated on FN, even at 500 ng/cm^2, fared no better than those seeded onto plates with no ECM coating (Fig. 1, *middle* and *bottom panels*). Similarly, when glioma cells were plated on a fixed ECM coating concentration of 500 ng/cm^2 but treated with escalating doses of topotecan, the proportion of cells manifesting apoptotic features at each dose of drug was significantly less in the VN-plated cells (Fig. 2, *row A*). For U251 cells, the median lethal dose (LD_{50}) on VN was ~130 ng/ml of topotecan, almost 4-fold higher than the LD_{50} on FN (35 ng/ml).

The superiority of VN over FN with regards to protection from drug-induced cell death was not a result of differences in cell adhesion on the two substrates. Moreover, because the inhibition of DNA replication by topotecan is S-phase specific, we evaluated the glioma cells by PI FACS to determine whether the protective effect of VN was associated with exit from cell cycle; no such alterations were noted (data not shown).

Cell Surface Integrin Receptors for VN Are Capable of Conferring Protection for Apoptosis. Although the data illustrated in Fig. 1 suggest a role for VN in enhancing the survival of glioma cells, they do not confirm that this effect is mediated by ligation receptors capable of binding VN, which include the $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins as well as the receptor for urokinase (urokinase plasminogen activator receptor; Ref. 16). To address this issue, we targeted the integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$, because they have been shown previously to play an important role in endothelial cell survival (8), using integrin-specific antibodies immobilized on culture dishes to determine whether the ligation of these integrins may confer enhanced survival to glioma cells. Cells were plated onto chamber slides coated with

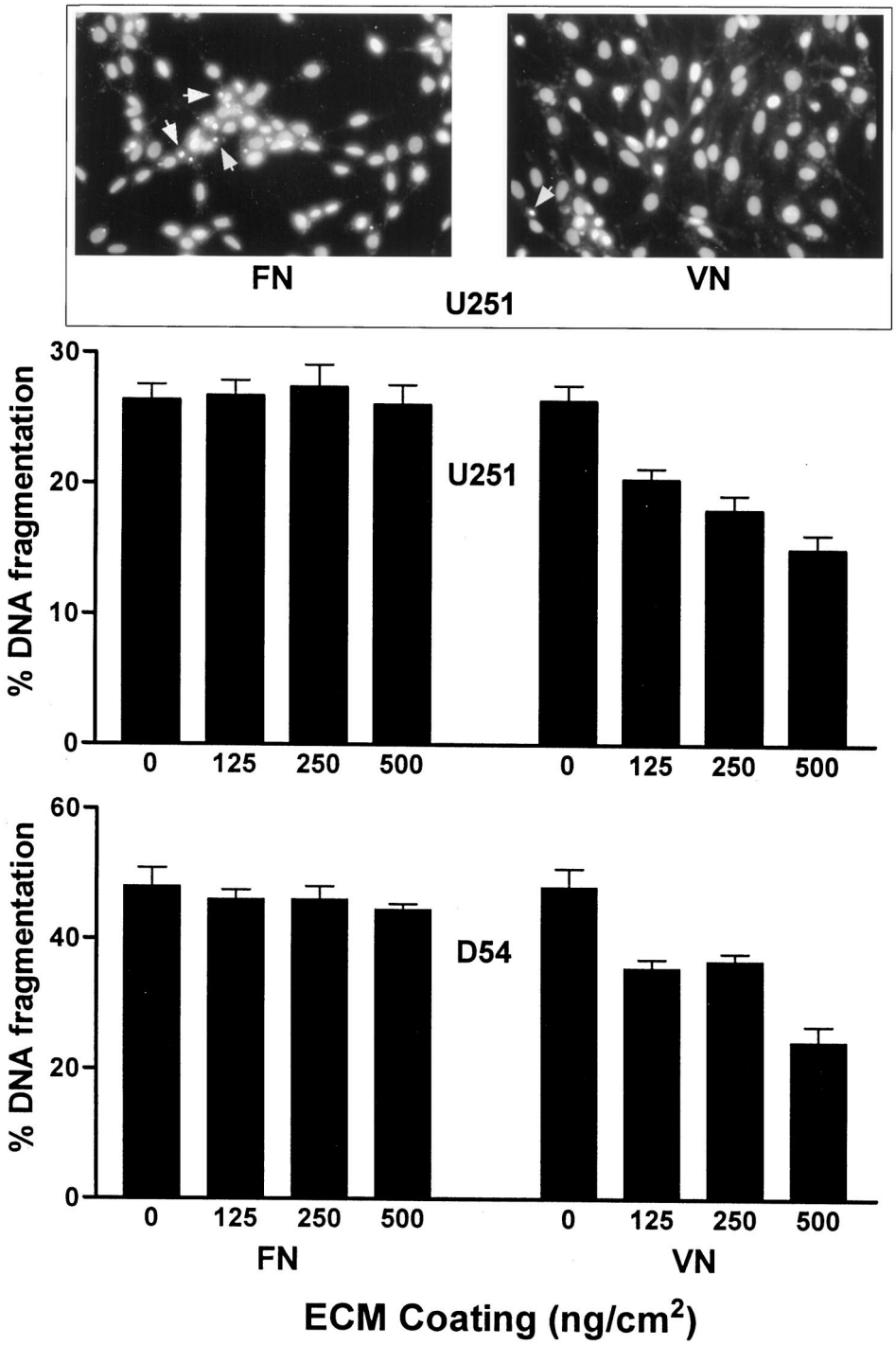

Fig. 1 VN protects glioma cells from drug-induced apoptosis in a dose-dependent manner. Glioma cells were seeded onto either FN or VN coatings and treated with topotecan (20 ng/ml). *Top panel,* glioma cells were assayed for DNA fragmentation using an *in situ* TUNEL technique. TUNEL labeling nuclei (small, bright nuclei marked by *arrows*) were first photographed, followed by double exposure for the background of PI-labeled nuclei (nonapoptotic, large oval nuclei). In addition to labeling by TUNEL, apoptotic nuclei tended to be smaller, fragmented, and eccentrically placed. Compared with the numerous TUNEL-positive cells on the FN substrate, relatively few cells were labeled with TUNEL on the VN substrate. *Middle* and *bottom panels,* a dose-dependent reduction in apoptosis was observed with both glioma cell lines plated on VN. In contrast, FN, even at high coating concentrations, had no significant effect on drug-induced apoptosis. All experiments were performed in triplicate; values are expressed as means; *bars,* SE.

monoclonal antibodies specifically directed toward the FN receptor ($\alpha_5\beta_1$ integrin) or the two VN receptors ($\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins) and then treated with a fixed dose of topotecan (20 ng/ml; Fig. 2, *row B*). Although both cell lines were confirmed to express the $\alpha_5\beta_1$ integrin (FN receptor) by immunofluorescence (data not shown), the antibody to the FN receptor produced only a marginal reduction in apoptosis in U251 cells, while demonstrating no significant effect in D54 glioma cells (Fig. 2, *row B*). This observation was in sharp contrast to U251 cells plated on either of the VN receptor antibodies (anti-$\alpha_v\beta_3$ or anti-$\alpha_v\beta_5$), where the proportion of apoptotic cells was reduced by >75% compared to cells seeded onto control IgG. Similarly, D54 cells seeded onto the anti-$\alpha_V\beta_5$ antibody coating, but not the anti-$\alpha_v\beta_3$ antibody coating, were protected from apoptosis.

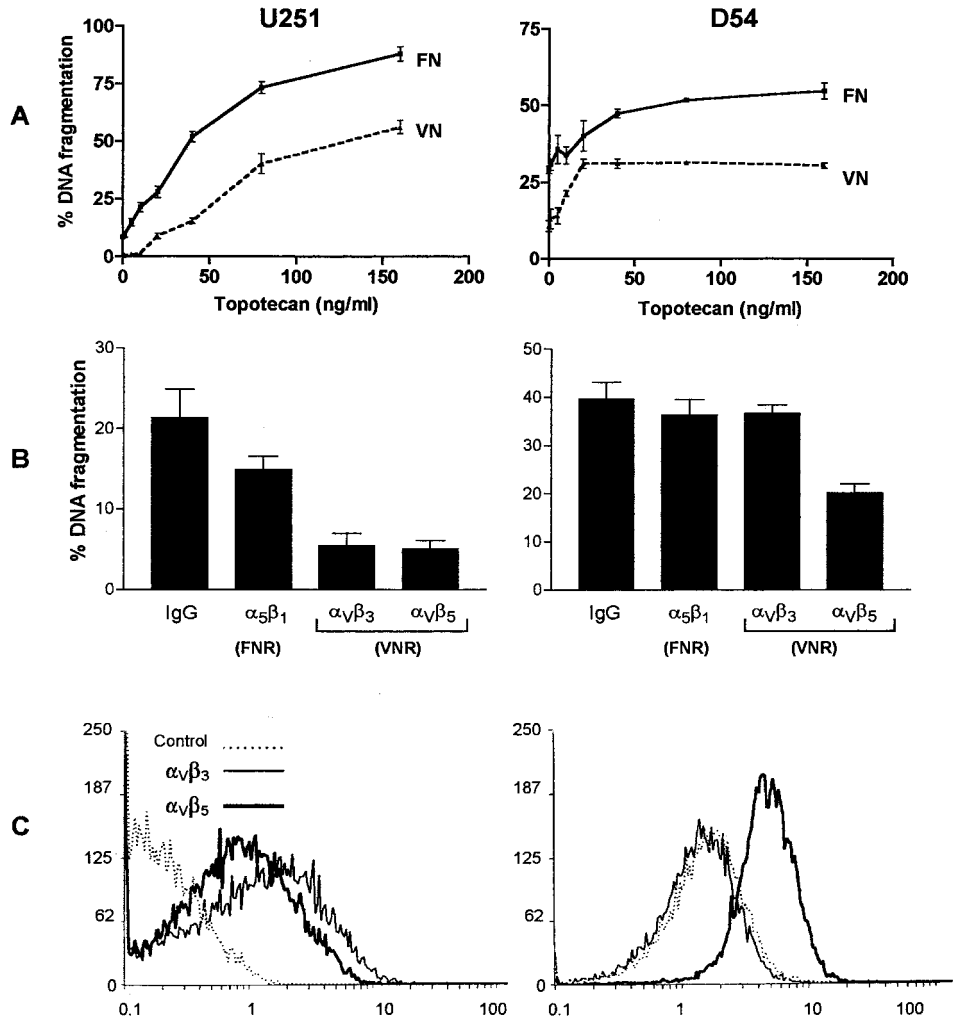

Fig. 2 The protective effect of VN is mediated by its cell surface receptors. *A*, U251 and D54 cells were plated onto chamber slides coated with 500 ng/cm^2 of either FN or VN and subsequently treated with increasing doses of topotecan. For both cell lines, a significantly lower proportion of VN-plated cells manifested evidence of apoptosis than did FN-plated cells. *B* and *C*, U251 and D54 cells were plated onto chamber slides coated with either mouse IgG (control) or monoclonal antibodies that ligate the FN receptor (*FNR*, $\alpha_5\beta_1$) or the VN receptors (*VNR*; $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins) and then exposed to 20 ng/ml of topotecan. Although both VN receptor antibodies conferred protection against apoptosis for U251 cells, only the $\alpha_V\beta_5$ antibody coating was protective for D54 cells (*B*). The reason for this difference is demonstrated in *C*, in which cell surface immunolabeling assessed by FACS revealed that U251 cells express both VN receptors, whereas only the $\alpha_V\beta_5$ subtype is expressed by D54 cells [FACS data pertain to VN-plated cells (500 ng/cm^2); similar results were observed with FN-plated cells (data not shown)]. All experiments were performed in triplicates, and values are expressed as means; *bars*, SE.

This enhanced cell survival on the immobilized antibody coatings corresponded to the expression of the respective cell surface integrins as determined by FACS; although U251 immunolabeled for both cell surface VN receptors, D54 was positive for only the $\alpha_v\beta_5$ integrin (Fig. 2, *row C*). Taken together, these results suggest that either of the two classic VN receptors is capable of mediating protection against apoptosis in glioma cells.

Inhibition of the Receptors for VN Leads to Increased Cell Death. To further corroborate a role for VN and VN receptors in enhancing glioma survival, a specific RGD peptide (cycRGD) was used to selectively antagonize the VN receptor. It is important to note that in contrast to previous experiments illustrated in Figs. 1 and 2, these experiments with VN receptor antagonistic RGD peptides (Fig. 3) were performed in the absence of topotecan. As illustrated in Fig. 3, both U251 and D54 glioma cells underwent increased apoptosis in response to treatment with cycRGD during adhesion to coverslips coated with FN and VN; the standard linear RGD peptide had no significant effect compared with the control RGE peptide treatment. At 100 μM, the cycRGD peptide is a relatively selective antagonist of VN receptors (Ref. 13; the threshold concentration required for inhibiting the FN receptor is close to an order of magnitude greater), whereas the linear RGD peptide at this concentration is significantly less effective as an inhibitor of the VN receptor integrin compared with cycRGD (6, 13). Thus, the specific antagonism of the VN receptor was associated with an increase in cell death.

VN Coatings Lead to Increased Expression of Two Antiapoptotic Proteins, Bcl-2 and Bcl-X_L. Because chemoresistance to a wide variety of anticancer agents have been associated with the overexpression of several antiapoptotic members of the Bcl family of proteins, such as Bcl-2 and Bcl-XL, we prepared lysates of glioma cells that were grown on either FN or VN and assayed the extracts for their levels of these two proteins. Furthermore, because the protective effect of Bcl-2 or Bcl-X_L can effectively be neutralized by their heterodimerization with proapoptotic counterparts such as Bax, the tumor cell lysates were also subjected to immunoblots for Bax levels to determine the net balance between the anti- and proapoptotic proteins, which are important determinants in the

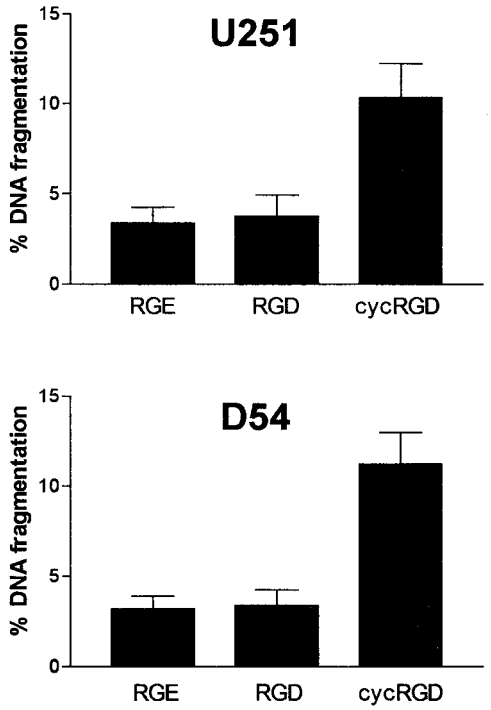

Fig. 3 A VN receptor-specific RGD peptide can increase glioma apoptosis. Treatment of glioma cells with a cycRGD (100 μM) that specifically targets the VN receptors led to increased apoptosis compared with the standard linear RGD or control RGE peptides. The increase in apoptosis associated with cycRGD peptide treatment was not a result of alterations in cell adhesion to coverslips (data not shown). Values are represented as means based on at least three separate experiments; *bars*, SE.

decision between cell survival and death, with the high Bcl-2:Bax or Bcl-X_L:Bax ratios typically favoring survival.

As illustrated in Fig. 4, both U251 and D54 cells plated on VN demonstrate a significant increase in the ratios for Bcl-2:Bax and Bcl-X_L:Bax, largely as a result of increases in levels of Bcl-2 and Bcl-X_L, because Bax expression remained relatively unchanged. It is interesting to note that both in the presence and absence of drug, the Bcl-2/Bax- as well as the Bcl-X_L:Bax ratios were greater in cells grown on VN compared with FN. As such, the up-regulation of antiapoptotic proteins such as Bcl-2 and Bcl-X_L may represent a possible mechanism by which VN confers enhanced survival to glioma cells.

DISCUSSION

Although the classic functions ascribed to proteins that comprise the ECM have been limited previously to those of cell adhesion and structural scaffolding, recent reports have expanded the functional spectrum of these proteins beyond their simple adhesive roles. In this regard, ECM proteins have been shown to interact with their cognate cell surface receptors (integrins) in a variety of nonneoplastic cell types to initiate signal transduction events that enhance cell survival by way of inhibiting apoptosis (7). Whether an ECM-integrin interaction could confer a survival advantage to neoplastic cells, such as malignant gliomas, in the setting of a chemotherapeutic challenge had not, however, been addressed previously. In this regard, we pursued the postulate that VN, an ECM protein whose expression is unique in the adult central nervous system at the interface where glioma cells encounter the normal brain parenchyma, may protect these tumor cells from apoptotic death induced by chemotherapeutic stress. To this end, we were able to demonstrate that VN is protective against drug-induced apoptosis in glioma cells and that this enhanced cell survival can be mediated by the cognate receptors for VN, because ligation of the classic receptors for VN ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) also inhibited apoptosis (Figs. 1 and 2).

With regards to the VN receptor, the ligation of the $\alpha_V\beta_3$ integrin on endothelial cells has been associated with an increase in the expression of the antiapoptotic protein, Bcl-2 (17). Given that the overexpression of Bcl-2 has been correlated with a chemoresistant phenotype in numerous peripheral tumors (18), we pursued the hypothesis that the survival advantage conferred by VN to glioma cells may also involve an increase in the levels of Bcl-2 expression. In concordance with this postulate, Bcl-2 levels were indeed found to be higher in lysates of both U251 and D54 glioma cells plated on VN, compared with those of FN-plated cells (Fig. 4). Moreover, VN-plated glioma cells demonstrated increased expression of another antiapoptotic protein, Bcl-X_L. The observed increases in the expression of these two antiapoptotic proteins may thus underlie the survival advantage observed with the VN substrate.

Bcl-2, Bcl-X_L, and other antiapoptotic members of the Bcl family of proteins exert their protective effect, in part, by inhibiting the mitochondrial release of cytochrome *c* (19, 20), a critical cofactor in the proteolytic activation of the caspases, the effector molecules of apoptosis. Evidence for a role for the Bcl family of proteins in the treatment-resistant phenotype in malignant gliomas has been demonstrated previously. Nagane *et al.* (21) have reported recently that the resistance of glioma cells that harbor the constitutively activated mutant epidermal growth factor receptor to cisplatinum therapy was a consequence of the up-regulation of Bcl-X_L. Moreover, the endogenous or ectopic overexpression of either Bcl-2 or Bcl-X_L in glioma cells has been shown to be able to abrogate programmed cell death induced by radiation (22) or chemotherapeutic agents, such as nitrosoureas (22), cisplatinum (21, 23), and agents that target various components of the growth factor signal transduction cascade (24–26). However, although the antiapoptotic role of Bcl-2 and Bcl-X_L in gliomas has been characterized, the regulation of these proteins by the ECM in the context of high-grade gliomas has not been assessed previously. In this report, we have demonstrated that VN, an ECM protein expressed specifically by glioma cells *in vivo*, increases the expression of these antiapoptotic proteins in glioma cells with a concomitant reduction in chemotherapy-induced programmed cell death (Fig. 4).

The mechanism by which the VN-VN receptor interaction modulates the expression of Bcl family proteins has been addressed in the context of endothelial cells, in which the ligation of the endothelial $\alpha_v\beta_3$ integrin is associated with a decrease in p53 levels (17). p53, when present, typically decreases Bcl-2 and increases Bax (27), thereby tipping the ratio in favor of Bax and thus cell death. Therefore, upon $\alpha_V\beta_3$ ligation, the reduced p53 expression observed in endothelial cells leads to the opposite situation, in which there is an increase in the Bcl-2:Bax

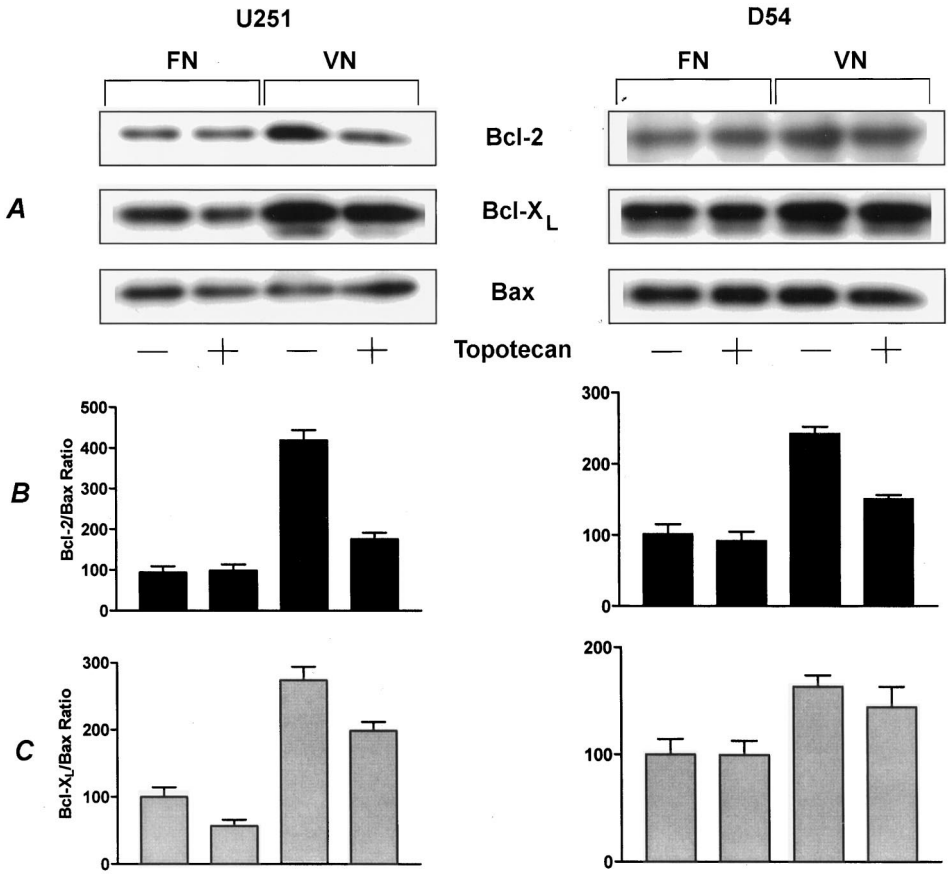

Fig. 4 Glioma cells grown on VN express higher levels of the antiapoptotic proteins, Bcl-2 and Bcl-X_L. Cells seeded onto ECM coatings were treated with either 20 ng/ml of topotecan (+) or vehicle (−) for 2 days under serum-free conditions, following which cell lysates were prepared and assayed for levels of two antiapoptotic proteins (Bcl-2 and Bcl-X_L) and their proapoptotic counterpart, Bax. Immunoblots show that although Bax levels do not fluctuate, levels of Bcl-2- and Bcl-X_L increase when the cells are grown on VN (representative blots in *A*), an effect that is most apparent in the absence of topotecan. Immunoblot signals were quantitated with a densitometer, and the signals for Bcl-2 and Bcl-X_L were normalized to Bax levels to obtain the ratios for the Bcl-2:Bax (■; *B*) and Bcl-X_L:Bax (▨; *C*). For each set of histograms, the Bcl-2:Bax or Bcl-X_L:Bax ratios derived from cells seeded on FN coatings in the absence of topotecan were designated arbitrarily as 100%. Histograms reflect at least three separate experiments with values representing means; *bars*, SE.

ratio, hence favoring cell survival (17). However, it is important to note that for the glioma cells evaluated in our present study, the p53 status for the D54 is wild type, whereas the p53 status in U251 is mutant. As both glioma cell lines demonstrated an increase in Bcl-2 expression when grown on VN (Fig. 4), our findings suggest that the increase in Bcl-2 levels and resistance to apoptosis conferred by VN in glioma cells may be independent of the p53 status.

The importance of the interaction between VN and its receptors in the survival of glioma cells is further supported by our observation that antagonism of the VN receptor sensitizes glioma cells to programmed cell death (Fig. 3). It is important to note that the increase in apoptosis after treatment with the VN receptor antagonist, cycRGD, occurred in the absence of a chemotherapeutic agent (*i.e.*, topotecan), further suggesting that the VN receptor itself may serve as a potential target for novel therapeutics. The potential therapeutic utility of VN receptor antagonism has been assessed in the setting of endothelial cells, which require the VN receptor ($\alpha_v\beta_3$) for cell survival. Treatment of animals with a neutralizing antibody against this integrin is able to induce massive apoptosis of proliferating endothelial cells that express this integrin and thereby induce regression of tumors *in vivo* by an antiangiogenic mechanism (8). More recently, peptidomimetic inhibitors of the $\alpha_v\beta_3$ integrin have been developed, and encouraging preclinical results of studies conducted with animals inoculated with germ cell tumors have been reported (28).

Although these anti-$\alpha_v\beta_3$ agents have been shown to be effective as monotherapy, they have yet to be evaluated in combination with other drugs. With respect to gliomas, the antagonism of the VN receptors, such as the $\alpha_v\beta_3$ integrin, may sensitize tumor cells to standard chemotherapeutic agents to which the cells were resistant previously. For example, as illustrated in Fig. 2*A*, the IC_{50} for topotecan-induced apoptosis for glioma cells plated on VN was 130 ng/ml, a drug concentration that is well beyond the cerebrospinal fluid levels that can be attained with the maximum tolerated dose of this drug (15), thereby effectively rendering these cells clinically resistant to this drug. In contrast, the IC_{50} of 35 ng/ml for glioma cells seeded on a non-VN substrate (FN) is achievable in the cerebrospinal fluid of test subjects. In this regard, the expression of VN by glioma cells selectively at the advancing tumor margin may underlie, in part, the observation that the great majority of tumor recurrences occur here, because the local deposition of VN may contribute to the chemoresistance of glioma cells in this location. Conversely, agents that disrupt the VN-VN receptor interaction may serve a potential role in sensitizing glioma cells to cytotoxic drugs.

In conclusion, we have presented data in this report that for the first time ascribes an antiapoptotic role to the VN-VN

receptor interaction in malignant gliomas. In this regard, the VN-integrin interaction was able to protect glioma cells from drug-induced cell death, with a concomitant increase in expression of Bcl-2 and Bcl-X_L, two key antiapoptotic proteins that have been associated with treatment resistance in a variety of tumor types, including gliomas. As an ECM molecule that may confer protection from apoptosis, VN expression by glioma cells at the tumor margin may underlie, in part, the relative chemoresistance of these cells. Moreover, as VN and its receptor have been shown to mediate glioma motility as well as the process of neovascularization, the recently reported agents that target this ECM-integrin interaction (28) may thus have a pleiotropic effect, potentially impacting upon multiple aspects of glioma biology that comprise motility/invasion and angiogenesis, as well as treatment resistance.

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