

# Suppression of p53 Activity and p21<sup>WAF1/CIP1</sup> Expression by Vascular Cell Integrin $\alpha$ v $\beta$ 3 during Angiogenesis

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## Abstract

Induction of p53 activity in cells undergoing DNA synthesis represents a molecular conflict that can lead to apoptosis. During angiogenesis, proliferative endothelial cells become apoptotic in response to antagonists of integrin  $\alpha$ v $\beta$ 3 and this leads to the regression of angiogenic blood vessels, thereby blocking the growth of various human tumors. Evidence is presented that administration of  $\alpha$ v $\beta$ 3 antagonists during angiogenesis in vivo selectively caused activation of endothelial cell p53 and increased expression of the p53-inducible cell cycle inhibitor p21<sup>WAF1/CIP1</sup>. In vitro studies revealed that the ligation state of human endothelial cell  $\alpha$ v $\beta$ 3 directly influenced p53 activity and the bax cell death pathway. Specifically, agonists of endothelial cell  $\alpha$ v $\beta$ 3, but not other integrins, suppressed p53 activity, blocked p21<sup>WAF1/CIP1</sup> expression, and increased the bcl-2/bax ratio, thereby promoting cell survival. Thus, ligation of vascular cell integrin  $\alpha$ v $\beta$ 3 promotes a critical and specific adhesion-dependent cell survival signal during angiogenesis leading to inhibition of p53 activity, decreased expression of p21<sup>WAF1/CIP1</sup>, and suppression of the bax cell death pathway. (*J. Clin. Invest.* 1996. 98:426–433). Key words: endothelial cells • gene expression • apoptosis • cell cycle • bcl-2

## Introduction

Angiogenesis is a critical process during development, wound healing, and various diseases including cancer, adult blindness, and inflammatory disorders (1). We recently showed that integrin  $\alpha$ v $\beta$ 3 becomes expressed on angiogenic vascular cells where it plays a critical role in angiogenesis induced by basic Fibroblast growth Factor (bFGF)<sup>1</sup>, TNF- $\alpha$ , or fragments of human tumors in the chick embryo (2, 3). In fact, antagonists of  $\alpha$ v $\beta$ 3 administered intravenously to chick embryos caused regression of angiogenic blood vessels on the chorioallantoic membrane (CAM) due to unscheduled apoptosis (2). Importantly, this resulted in the regression of human tumors on the chick CAM or in human skin transplants on SCID mice (2, 4).

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1. *Abbreviations used in this paper:* bFGF, basic fibroblast growth factor; CAM, chorioallantoic membrane; EMSA, electrophoretic mobility shift assay; HUVEC, human umbilical vein endothelial cells; TNA, total nucleic acids.

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Cell survival and proliferation depend on appropriate signals mediated by growth factors and/or adhesion proteins within the extracellular matrix (5, 6). However, incompatible signaling events can result in apoptosis (7–12). We hypothesized that during angiogenesis, integrin  $\alpha$ v $\beta$ 3 promotes an adhesion-dependent survival signal necessary for normal cell cycle progression, since antagonists of  $\alpha$ v $\beta$ 3 administered in vivo preferentially caused apoptosis of proliferating vascular cells (2). Therefore, we examined whether the observed apoptosis might be associated with the induction of conflicting signals during endothelial cell cycle progression. Recent reports have demonstrated that cells expressing active p53 while undergoing DNA synthesis in vitro become apoptotic, presumably due to conflicting signals (7–11). Induction of apoptosis by p53 has been associated with its ability to promote or repress transcription of genes that either influence cell cycle progression or directly regulate cell survival and apoptosis (13–16). Therefore, experiments were designed to evaluate whether ligation of endothelial cell  $\alpha$ v $\beta$ 3 influenced expression and/or activity of p53 and of putative mediators of p53-induced apoptosis during angiogenesis.

## Methods

*Cell lines and tissue culture.* Human umbilical vein endothelial cells (HUVECs) from pooled donors (Clonetics Corp., San Diego, CA) were grown in M199 containing 20% FBS, 100  $\mu$ g/ml gentamicin, 4 mM L-glutamine, 0.9 mg/ml heparin and 30  $\mu$ g/ml endothelial cell growth supplement (Upstate Biotech. Inc., Lake Placid, NY).

*Cell adhesion to antiintegrin antibodies as agonists of integrin function.* For evaluation of effects by individual integrins, plastic dishes were first coated with 25  $\mu$ g/ml goat anti-mouse polyclonal antibodies (Jackson ImmunoResearch Labs., Inc., West Grove, PA) at 37°C for 2 h, followed by blocking with 1% heat-denatured BSA at 37°C for 1 h. Then, mouse monoclonal antibodies (10  $\mu$ g/ml) LM 609 (anti- $\alpha$ v $\beta$ 3), P1F6 (anti- $\alpha$ v $\beta$ 5), or P4C10 (anti- $\beta$ 1) were allowed to bind the immobilized anti-mouse antibody for 14 h at 4°C. Subconfluent (~50%) HUVEC cells were harvested with EDTA, washed, and resuspended in 20 mM Hepes, 140 mM NaCl, 5 mM KCl, 1 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 5 mM glucose, and 5 mM sodium pyruvate, pH 7.4 (adhesion buffer). Cells were then allowed to attach to antibody-coated dishes for 4 h at 37°C. To maintain cells in suspension, some dishes were coated only with 1% heat-denatured BSA.

*Chick CAM angiogenesis assay.* 10-d-old chick embryos, purchased from McIntyre Poultry (Lakeside, CA), were incubated at 37°C with 60% humidity. Filter discs (Whatman Inc., Clifton, NJ), 1  $\times$  1 cm, were saturated with 1  $\mu$ g/ml bFGF (Genzyme Corp., Cambridge, MA) and placed on top of an avascular zone of the CAM as previously described (3). After 20–24 h, at the peak of  $\alpha$ v $\beta$ 3 expression (S. Strömblad, and D.A. Cheresh, unpublished results), embryos were injected intravenously with 100  $\mu$ l PBS containing antagonists to  $\alpha$ v $\beta$ 3, 300  $\mu$ g mAb LM 609 or 100  $\mu$ g peptide 66203 (cyclo-RGDFV). Control embryos received 300  $\mu$ g mAb CSAT (anti- $\beta$ 1) or 100  $\mu$ g peptide 69601 (cyclo-RADfV). 48 h later, CAM tissues directly underlying the filters were resected for isolation of nucleic acids or protein. Chick thymus was isolated from 8-wk-old chickens and used as positive control tissue.

**Isolation of CAM cells and sorting of endothelial cells.** After induction of angiogenesis, CAMs were resected and washed three times in sterile PBS, finely minced, and resuspended in 0.25% bacterial collagenase (Worthington Biochemical Corp., Freehold, NJ). After incubation for 120 min at 37°C with occasional vortexing, cells were washed three times in PBS with 1% BSA, and then incubated with a 1:100 dilution of antiFactor VIII rabbit polyclonal antibodies 016P (BioGenex Labs., San Ramon, CA) at room temperature for 60 min. After three washes in PBS/1% BSA, cells were incubated with FITC-conjugated goat anti-rabbit antibodies at 1:50 dilution (Biosource International, Camarillo, CA) for 45 min at 25°C. Cells were then washed three times (PBS/1% BSA) and positive staining cells were separated from negative by flow cytometry using a FACStar® cell sorter (Becton Dickinson & Co., Mountain View, CA) with Consort 30 software (Becton Dickinson & Co.). Cells incubated only with the secondary antibody were used to set negative markers and the sortings typically yielded 15–20% positive cells.

**Preparation of nucleic acids and mRNA analyses.** Total nucleic acids (TNA) were isolated by the method of Durnam and Palmiter (17) using 3–5 pooled CAMs per preparation. The TNA concentrations were measured by spectrophotometry at 260 nm. Chicken bcl-2 mRNA and  $\beta$ -actin mRNA was quantified by solution hybridization where 50,000 cpm of a <sup>35</sup>S-CTP-labeled (Amersham Corp., Arlington Heights, IL) probe was hybridized to 1–2  $\mu$ g ( $\beta$ -actin) or 10–20  $\mu$ g (bcl-2) of the TNA samples as previously described (18) with reagents from Promega Biotech. Inc. (Madison, WI). The bcl-2 clone used was a 532-bp *Ava*I-*Bam*HI (from 71 to 603) cDNA fragment of the chicken bcl-2 (19), subcloned into a pGEM™ 4Z riboprobe vector (Promega Biotech. Inc.).  $\beta$ -Actin mRNA was quantified using a 2.2-kb cDNA coding for chicken  $\beta$ -actin (20), subcloned into pGEM™ 3. The hybridization signals were compared with that of a standard curve produced using synthetic mRNA strands complementary to the probes made from the same cDNAs. Three TNA preparations were analyzed for each group and the values were determined using four incubations for each sample at two different concentrations, to ensure that the analyses were within a linear range.

**Western blot analyses.** Cells or tissues were lysed in a modified RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1% Triton X-100, 1% deoxycholic acid, 1 mM PMSF, with addition of Complete™ protease inhibitor cocktail [Boehringer Mannheim, Mannheim, Germany]). Protein concentrations were determined by a BCA assay (Pierce Chemical Co., Rockford, IL) using BSA as a standard. Cell lysates from three to four pooled CAMs or from HUVEC cells (30  $\mu$ g protein) were run in an 8% (p53) or 12% (all others) SDS-PAGE, including Rainbow™ molecular weight size markers (Amersham Corp.) under DTT-reduced (50 mM) or non reduced (chicken bcl-2) conditions. Proteins were then transferred to a nitrocellulose filter by semidry blotting in 25 mM Tris, 200 mM glycine, 1.3 mM SDS, 20% methanol, pH 8.3, at 160 mA for 3 h. The filters were stained with 10% Ponceau S solution (Sigma Chemical Co., St. Louis, MO) for 10 min to verify equal loading and transfer efficiency (data not shown). The filters were then blocked in 10% dry milk for 14 h at 4°C and probed with 1–2  $\mu$ g/ml antibodies sc 492 (anti-bcl-2), sc 493 (anti-bax) (Santa Cruz Biotech., Santa Cruz, CA), anti-p53 polyclonal antibody-7 (Oncogene Science Inc., Cambridge, MA), or anti-p21<sup>WAF1</sup> polyclonal antibody-5 (Oncogene Science Inc.) for 60 min at RT. After washes in TBS-T (20 mM Tris, 137 mM NaCl, 0.1% Tween 20), signals were detected by horseradish peroxidase-conjugated secondary goat anti-rabbit antibodies at 1:3,000 dilution (Bio-Rad Laboratories, Richmond, CA) or rabbit anti-sheep at 1:5,000 dilution (Oncogene Science Inc.) for 45 min at RT. After extensive washing in TBS-T, electrochemical luminescence was developed (Amersham Corp.). Membranes were then exposed to X-omat AR x-ray film (Eastman Kodak Co., Rochester, NY) and quantitative values were determined using a Personal Densitometer (Molecular Dynamics, Inc., Sunnyvale, CA) coupled to ImageQuant software (Molecular Dynamics, Inc.).

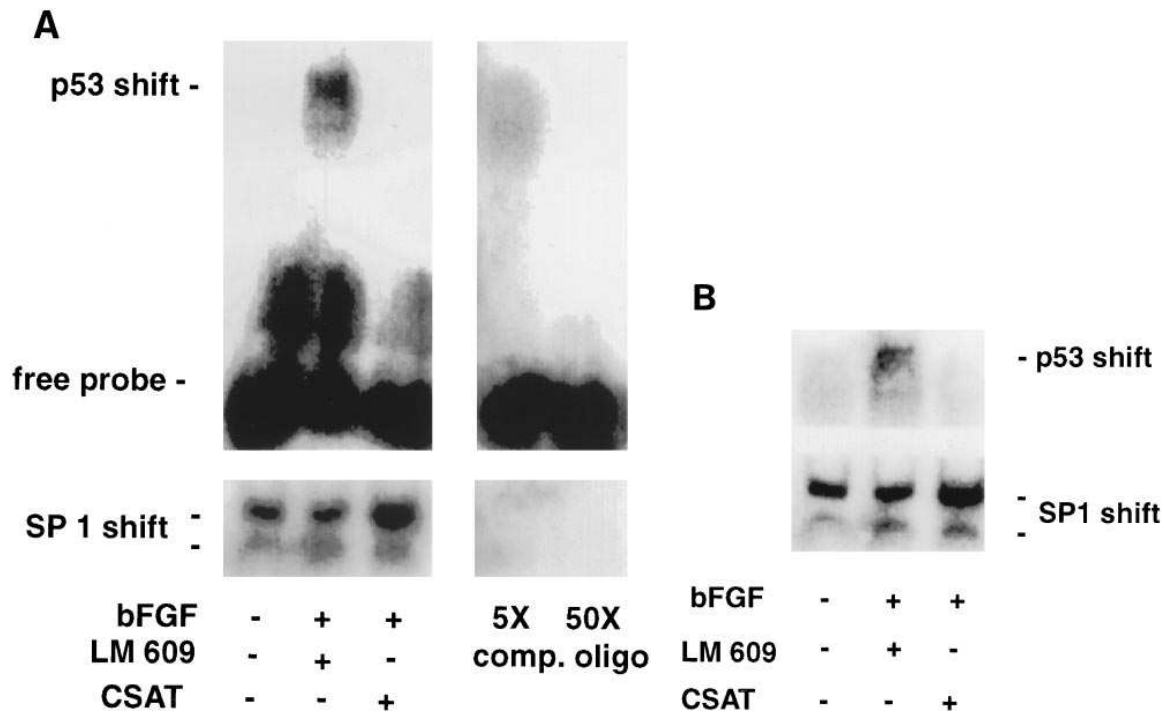
**Electrophoretic mobility-shift assays (EMSA).** Nuclear extracts were

isolated from  $2 \times 10^5$  to  $1 \times 10^6$  cells by the method of Schreiber et al. (21). Cells were suspended in 400  $\mu$ l cold buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) and allowed to swell on ice for 15 min; after which, 25  $\mu$ l 10% Nonidet P-40 was added and the tubes extensively vortexed. After centrifugation, the nuclear pellet was resuspended in 80  $\mu$ l ice cold buffer B (20 mM Hepes, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) and vigorously rocked at 4°C for 15 min. Nuclear extracts were cleared by centrifugation. Protein content was assayed with Bradford reagent (Bio-Rad Laboratories). <sup>32</sup>P-end-labeled double-stranded oligonucleotides containing the following human recognition sequences were used: p53 (5' CAG GCA TGT CTA CAG GCA AAG GCA TGT CTG 3') and SP1 (5' ATT CGA TCG GGG CGG GGC GAG C 3'). An oligonucleotide containing a mutated p53 recognition sequence (5' CAT CAA TGT CTA CAG GCA AAG GCA TAC GTG 3') served as a control to measure nonspecific binding of proteins present in the nuclear extracts. Each reaction mixture contained 10 fmol probe and 5  $\mu$ g protein from nuclear extracts. Binding reactions were conducted in 50 mM Hepes, 30% glycerol, 50 mM KCl, 10 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM DTT, and 1 mg poly(dI-dC) in a final volume of 15  $\mu$ l. These reactions were allowed to occur at 4°C for 20 min. For supershift experiments, nuclear extracts were preincubated with 1  $\mu$ g of anti-p53 monoclonal antibody 421 (Oncogene Science Inc.) for 20 min at 4°C. Complexes were resolved on 6% nondenaturing polyacrylamide gels in 50 mM Tris, 45 mM borate, and 0.5 mM EDTA, pH 7.4. The samples were then subjected to electrophoresis for 2 h at 10V/cm. Gels were dried and subsequently visualized with a PhosphorImager (Molecular Dynamics, Inc.).

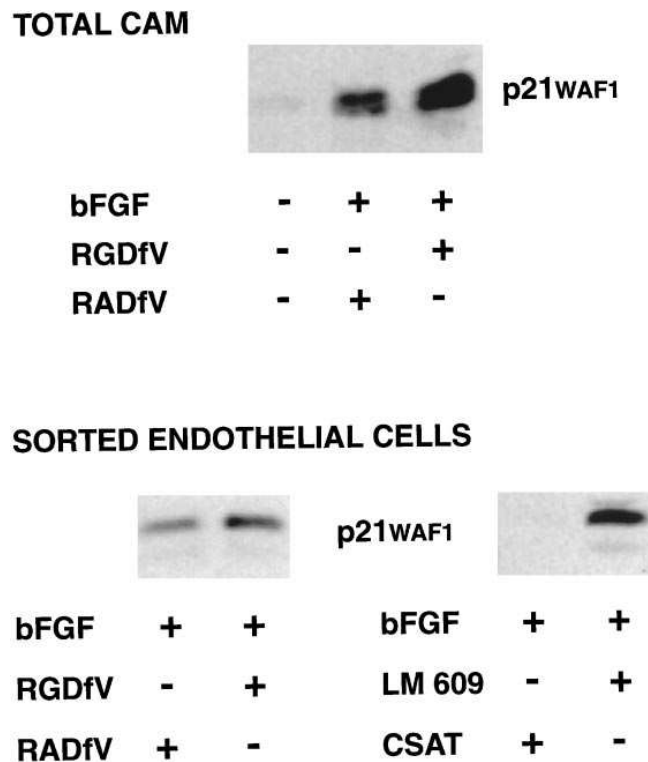
## Results

Experiments were designed to investigate the role of integrin  $\alpha$ v $\beta$ 3 in the regulation of vascular cell survival during angiogenesis. Angiogenesis was induced with bFGF on the chick CAM of 10-d-old chick embryos. 1 d later embryos were injected intravenously with anti- $\alpha$ v $\beta$ 3 monoclonal antibody (mAb LM609) or a control antibody directed to chick  $\beta$ 1 integrins (mAb CSAT). Administration of anti- $\alpha$ v $\beta$ 3 specifically leads to extensive apoptosis among proliferating endothelial cells on these CAMs within 48 h (6). To evaluate whether the apoptosis observed was associated with induction of p53 activity, nuclear extracts prepared from these CAMs were examined for p53 binding activity in a gel mobility shift assay. Administration of a single intravenous dose of anti- $\alpha$ v $\beta$ 3, sufficient to induce apoptosis after bFGF stimulation (2), caused a marked increase in p53 DNA binding activity as measured by the mobility shift (Fig. 1 A). However, CAMs examined from embryos injected with anti- $\beta$ 1 showed negligible apoptosis (2) and p53 activity (Fig. 1 A). The induction of p53 activity with anti- $\alpha$ v $\beta$ 3 was specific since excess unlabeled oligonucleotide effectively competed for this binding activity (Fig. 1 A) and a mutant oligonucleotide failed to cause the gel shift (data not shown). Also, injection of anti- $\alpha$ v $\beta$ 3 produced no change in SP1 binding activity (Fig. 1 A).

To determine whether this increased p53 activity was associated with endothelial cells within these tissues, single cell suspensions isolated from CAMs by collagenase treatment were stained for the endothelial cell marker, Factor VIII, and were sorted by flow cytometry. Analysis of nuclear extracts from these CAM-sorted endothelial cells revealed increased p53 binding activity after exposure to both bFGF and anti- $\alpha$ v $\beta$ 3 while SP1 binding activity remained unchanged (Fig. 1 B). In contrast, injection of mAb CSAT had no effect on p53 or SP1



**Figure 1.** Regulation of p53 activity during bFGF-induced angiogenesis on the chick CAM. (A) Angiogenesis was stimulated with bFGF on the chick CAM in the presence of anti- $\alpha$ v $\beta$ 3 or anti- $\beta$ 1. Nuclear extracts from three pooled CAMs were analyzed by an EMSA, examining p53 or SP1 DNA binding activity as described in Methods (left). In parallel, the nuclear extract from the anti- $\alpha$ v $\beta$ 3 treated CAM was incubated with a 5- or 50-fold excess of unlabeled oligonucleotide (right). (B) Angiogenesis was stimulated on the CAM with bFGF for 3 d in the presence of anti- $\alpha$ v $\beta$ 3 or anti- $\beta$ 1. Factor VIII-positive cells (endothelial cells) were then sorted out from a single cell suspension from six to ten pooled CAMs using a FACS<sup>®</sup> sorter as described in Methods. Nuclear extracts from the sorted endothelial cells were subsequently analyzed by p53 or SP1 EMSAs.

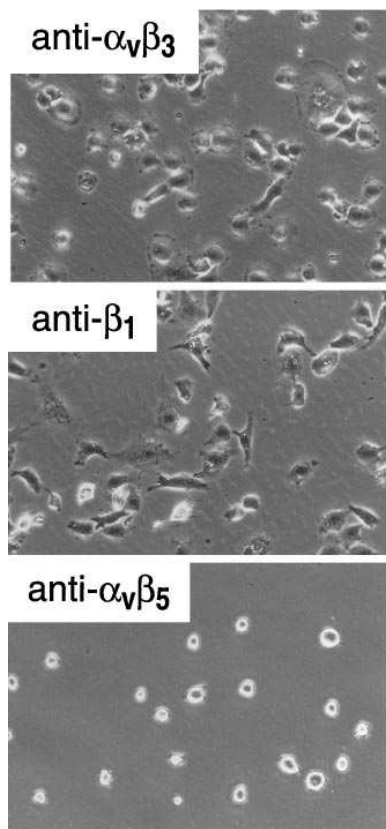


activity, consistent with its inability to affect angiogenesis or apoptosis (2). The observed gel shifts for p53 and SP1 were abolished with excess unlabeled oligonucleotide (data not shown). These findings demonstrate that administration of  $\alpha$ v $\beta$ 3 antagonists in vivo promote apoptosis and p53 activity among angiogenic endothelial cells.

Induction of apoptosis by p53 has been associated with its ability to promote transcription of genes that influence cell cycle progression. For example, p53 is known to induce expression of p21<sup>WAF1/CIP1</sup>, which binds to and inactivates cyclin/Cdk complexes and the proliferating cell nuclear antigen, thereby arresting cells in G1 (22–24). In fact, it was recently shown that overexpression of p21<sup>WAF1/CIP1</sup> promoted apoptosis of proliferating mammary epithelial cells in vitro, demonstrating a direct role for p21<sup>WAF1/CIP1</sup> in the apoptosis of proliferative cells (12).

To evaluate whether the induction of p53 activity was associated with increased expression of p21<sup>WAF1/CIP1</sup>, embryos un-

**Figure 2.** Regulation of p21<sup>WAF1/CIP1</sup> during bFGF-induced angiogenesis. Angiogenesis was stimulated on the CAM with bFGF for 3 d in the presence of anti- $\alpha$ v $\beta$ 3 antibodies,  $\alpha$ v $\beta$ 3 antagonist cyclic peptide RGDfV, anti- $\beta$ 1 antibodies or control cyclic peptide RADfV. Lysates from six to ten pooled CAMs (15  $\mu$ g protein) (top) or endothelial cells derived from these CAMs by FACS sorting (8  $\mu$ g protein) (bottom) were analyzed for p21<sup>WAF1/CIP1</sup> protein levels by Western blotting as described in Methods.



**Figure 3.** Endothelial cell attachment to immobilized antibodies. HUVECs were plated under serum free conditions on immobilized mAbs LM 609 (anti- $\alpha_v\beta_3$ ), P4C10 (anti- $\beta_1$ ), or PIF6 (anti- $\alpha_v\beta_5$ ) as described in Methods. Cells were photographed (100 $\times$ ) after 4 h of attachment.

dergoing angiogenesis in response to bFGF were injected with antagonists of  $\alpha_v\beta_3$ . As shown in Fig. 2, administration of antibody or cyclic peptide antagonists of  $\alpha_v\beta_3$  during angiogenesis caused a marked increase in p21<sup>WAF1/CIP1</sup> protein expression associated with CAM endothelial cells (Fig. 2). These findings suggest that during bFGF-induced angiogenesis in vivo, proliferating endothelial cells depend on a distinct integrin-mediated survival signal associated with inhibition of p53 activity and decreased expression of the cell cycle suppressor p21<sup>WAF1/CIP1</sup>.

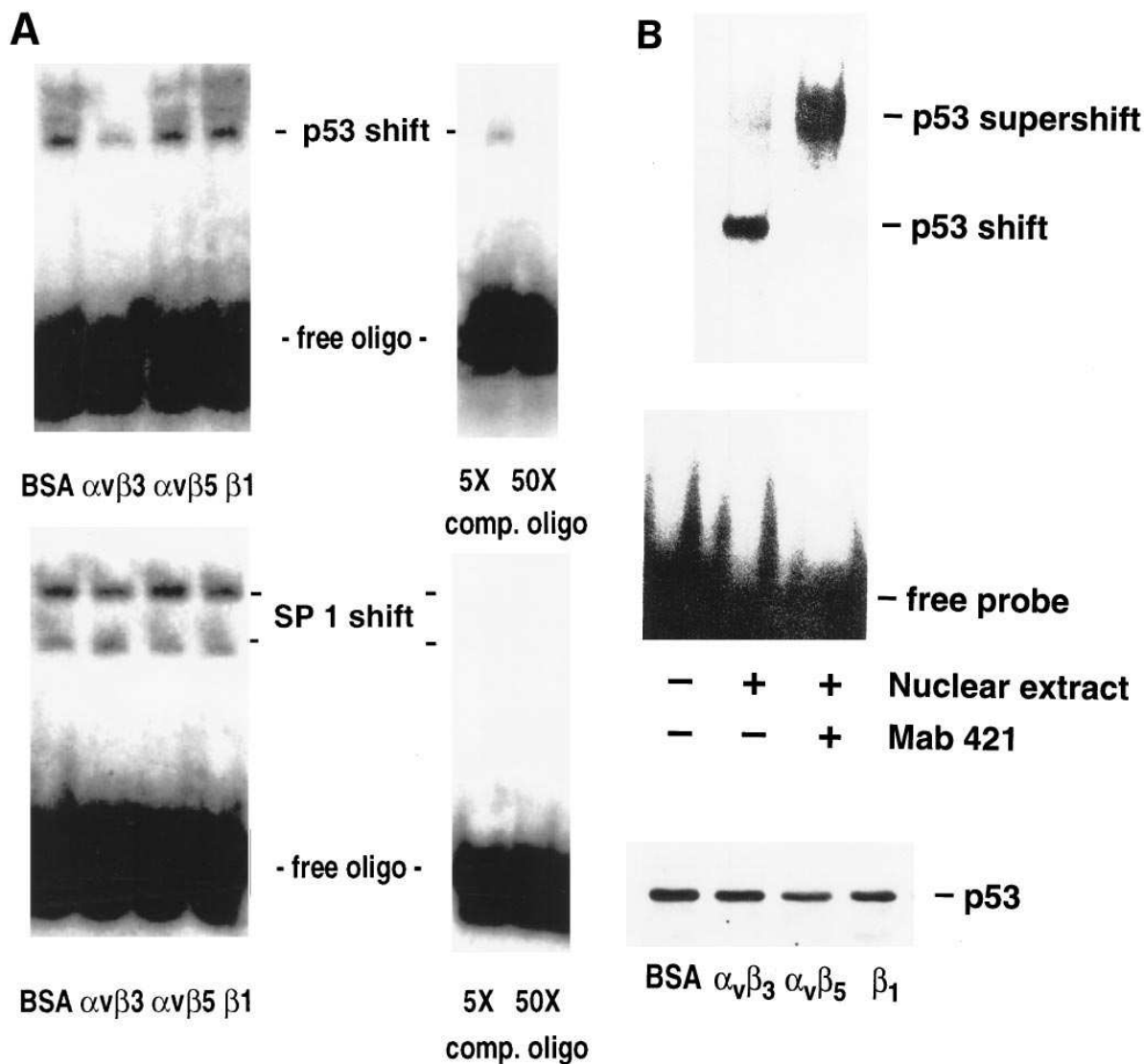
To examine whether ligation of  $\alpha_v\beta_3$  is sufficient to suppress p53 activity, human umbilical vein endothelial cells were plated in serum-free conditions on a substrate consisting of immobilized antiintegrin antibodies. When these antibodies are immobilized on a substrate, they are able to mediate integrin clustering at sites of focal adhesion, thereby acting as agonists, as previously described (25). This is opposed to the function of the same antibodies when used in solution or in soluble form in vivo, where they function as antagonists, blocking the integrin function (2, 3). As shown in Fig. 3, endothelial cells attach and spread on immobilized anti- $\alpha_v\beta_3$  or anti- $\beta_1$ , and attach yet fail to spread on anti- $\alpha_v\beta_5$ . We then examined nuclear extracts of these adherent endothelial cells for p53 DNA binding activity. As expected, there was little if any detectable p53 activity among adherent, proliferative cells maintained in serum containing growth medium (data not shown). However, when cells were plated on the nonadhesive substrate BSA under serum-free conditions, p53 activity was induced (Fig. 4 A) consistent with the induction of apoptosis among nonadherent cells (26–30). Interestingly, p53 activity was also induced in cells attached to either immobilized anti- $\beta_1$  or anti- $\alpha_v\beta_5$  (Fig. 4 A). In contrast, cells attached to immobilized anti- $\alpha_v\beta_3$  showed negligible p53 activity, suggesting this particular adhesion

event suppressed p53 activity. These results support the in vivo findings that integrin  $\alpha_v\beta_3$  ligation specifically influences the functional activity of p53. Importantly, regulation of p53 was independent of cell shape and actin assembly since immobilized anti- $\beta_1$  caused cell spreading yet failed to influence p53 activity. Once again, the specificity of the p53 gel shift was shown by the failure of a mutant oligonucleotide to induce a shift (data not shown), inhibition with excess unlabeled oligonucleotide, and the fact that SP1-binding activity was identical on all substrates (Fig. 4 A). In addition, the p53 activity was identified in an antibody-mediated supershift assay (Fig. 4 B). Surprisingly, the observed change in p53 DNA binding activity could not be explained by an altered level of p53 protein in these cells since p53 protein levels remained constant under all adhesion conditions (Fig. 4 B). These findings demonstrate that ligation of endothelial cell  $\alpha_v\beta_3$  is sufficient to inhibit p53 activity without influencing its expression. Furthermore, in support of the in vivo findings,  $\alpha_v\beta_3$  ligation promotes decreased p21<sup>WAF1/CIP1</sup> levels in these cells, revealing the association between this cell cycle suppressor and the activity of p53 during  $\alpha_v\beta_3$ -mediated adhesion of endothelial cells (Fig. 5). Thus, the ligation state of endothelial cell  $\alpha_v\beta_3$  regulates p53 activity and the expression of p21<sup>WAF1/CIP1</sup> both in vitro and in vivo, which may account for the role of  $\alpha_v\beta_3$  in vascular cell survival (2).

The role of p53 in promoting apoptosis has also been linked to its ability to repress transcription of bcl-2 while activating that of bax (14–16). Bcl-2 is known to potentiate cell survival based on its ability to dimerize with the death-promoting molecule bax (31–32). Therefore, cell survival is favored by a high bcl-2/bax ratio. To examine the role of endothelial cell  $\alpha_v\beta_3$  ligation on the bcl-2/bax ratio, cells were allowed to attach to immobilized antiintegrin antibodies as described above. While  $\alpha_v\beta_3$  and  $\beta_1$  integrin ligation caused increased bcl-2 expression relative to nonadherent cells, only  $\alpha_v\beta_3$  produced a concomitant decrease in bax expression, resulting in a sharp increase in the bcl-2/bax ratio (Fig. 6). These findings provide further support that ligation of endothelial cell  $\alpha_v\beta_3$  provides a distinct adhesion-dependent survival signal and supports our in vivo observations that antagonists of this integrin selectively promote endothelial cell apoptosis during angiogenesis. Consistent with this hypothesis is the fact that administration of antibody or peptide antagonists of  $\alpha_v\beta_3$  during chick CAM angiogenesis blocks bFGF-induced bcl-2 mRNA and protein expression on the CAM as measured by solution hybridization and Western blot analyses (Fig. 7).

## Discussion

Integrin-mediated cell attachment regulates cell survival and proliferation in several cell types in vitro (2, 5, 6, 12, 26–30). Also, cell attachment to extracellular matrix proteins in vitro is capable of regulating the apoptosis-related genes interleukin-1- $\beta$ -converting enzyme and bcl-2 (29, 30), as well as the cell cycle inhibitors p21<sup>WAF1/CIP1</sup> and p27<sup>KIP1</sup> (33). However, in vivo cells typically use multiple integrins for attachment to a wide variety of adhesive proteins. Thus, it remains unclear as to how individual matrix proteins or integrin receptors impact the cell survival within the context of a physiologically relevant extracellular matrix. To this end, we observed that integrin  $\alpha_v\beta_3$ , whose expression is enhanced on angiogenic vascular cells, promotes a survival signal since antagonists of this integrin



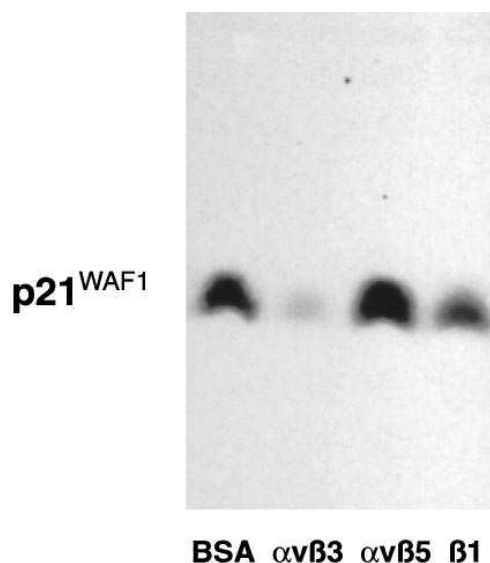
**Figure 4.** Integrin regulation of endothelial cell p53 activity. (A) Nuclear extracts were prepared from HUVECs attached to immobilized mAbs LM 609 (anti- $\alpha v\beta 3$ ), P4C10 (anti- $\beta 1$ ), or PIF6 (anti- $\alpha v\beta 3$ ) or maintained in suspension by BSA blocking for 4 h. Nuclear extracts were then used in p53 or SP1 EMSAs as described in Methods (left). HUVECs grown in serum-containing media were used as control. Extracts from HUVECs denied attachment were incubated with the  $^{32}\text{P}$ -labeled probe in the presence of 5- or 50-fold excess of unlabeled oligonucleotide (right). (B) (Top) Nuclear extract prepared from HUVECs that were prevented from adhering with heat-denatured BSA was analyzed in a super shift gel assay after preincubation in the presence or absence of anti-p53 mAb 421. (Bottom) Lysates (15  $\mu\text{g}$ ) from HUVECs attached to various immobilized antiintegrin antibodies were analyzed for p53 protein levels by Western blot analysis as described in Methods.

cause unscheduled apoptosis of newly forming blood vessels (2). This results in the disruption of ongoing angiogenesis or neovascularization in the quail embryo, chick CAM, rabbit eye, or human skin transplants on the SCID mouse (2–4, 34, 35). In fact, this leads to either prevention of human tumor growth or regression of preexisting tumors in the chick CAM or in human skin transplanted on SCID-mice (2, 4).

Integrin  $\alpha v\beta 3$  can recognize several extracellular matrix proteins, including vitronectin, fibronectin, osteopontin, von Willebrand factor, fibrinogen, and proteolyzed collagen (27, 36, 37). At present, it remains unclear as to which of the  $\alpha v\beta 3$ -directed ligand(s) promotes vascular cell survival during angiogenesis. However, recent work in our laboratory demonstrates that the matrix metalloproteinase-2 directly binds to  $\alpha v\beta 3$  and thereby associates with the surface of angiogenic vascular cells (38). Thus, matrix metalloproteinase-2 once bound to  $\alpha v\beta 3$ ,

may serve to promote endothelial cell invasion while providing a survival factor, i.e., proteolyzed collagen which binds to  $\alpha v\beta 3$ , thereby facilitating angiogenesis. In support of this contention,  $\alpha v\beta 3$  recognition of proteolyzed collagen has been shown to promote survival of melanoma cells in vitro (27).

Based on the fact that apoptosis was induced selectively in proliferating vascular cells upon treatment with antagonists to  $\alpha v\beta 3$  (2), we hypothesized that ligation of  $\alpha v\beta 3$  during angiogenesis was required for normal cell cycle progression. We found that ligation of  $\alpha v\beta 3$  during angiogenesis suppresses the expression and/or activity of p53 and the p53-inducible cell cycle inhibitor p21<sup>WAF1/CIP1</sup>. Presence of either p53 or p21<sup>WAF1/CIP1</sup> in proliferating cells has been found to induce apoptosis (7–11, 12). Thus, it appears that molecules that can promote cell cycle arrest cause apoptosis under other circumstances. The induction of apoptosis during proliferation is presumably caused by



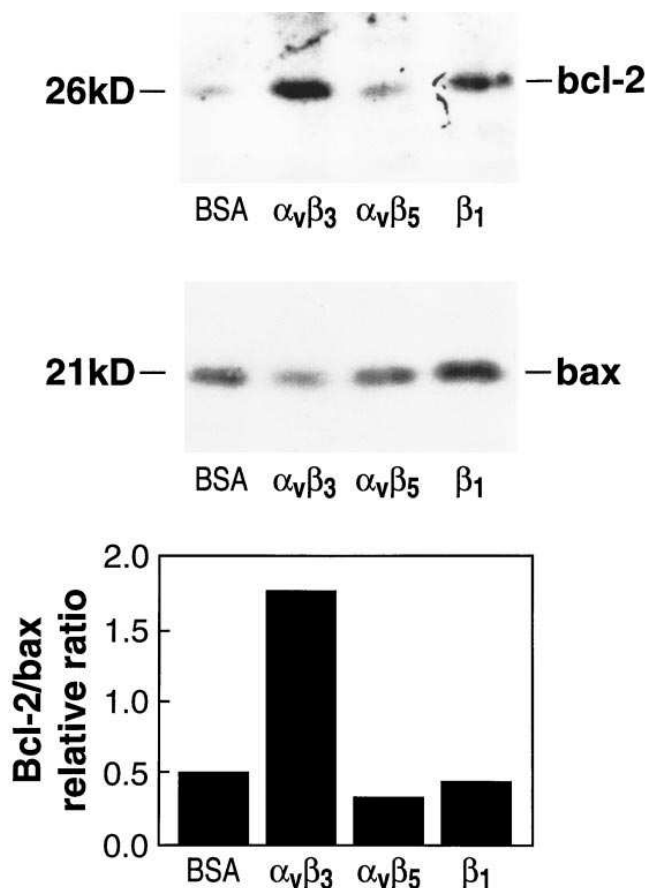
**Figure 5.** Integrin regulation of p21<sup>WAF1/CIP1</sup> in endothelial cells. HUVECs were plated under serum free conditions on immobilized mAbs LM 609 (anti- $\alpha$ v $\beta$ 3), P4C10 (anti- $\beta$ 1), or PIF6 (anti- $\alpha$ v $\beta$ 5) or were denied attachment on a BSA-coated surface for 4 h. Cells were then collected and lysates were analyzed for p21<sup>WAF1/CIP1</sup> protein levels by Western blotting as described in Methods.

incompatible molecular signals when stimulation of DNA synthesis occurs concurrent with growth arrest signals.

Furthermore, antagonists of  $\alpha$ v $\beta$ 3 administered during angiogenesis specifically prevented an increase in the bcl-2 levels, consistent with the role of p53 as a transcriptional repressor of bcl-2 (14, 15) and with the induction of vascular cell apoptosis. This is consistent with previous studies showing that lack of attachment to the extracellular matrix results in apoptosis of various cell types (2, 12, 26–30, 39, 40). It has been suggested that this phenomenon is caused by the lack of attachment per se (28), but other studies, including data presented here, clearly point out that cell survival is mediated through distinct signals from specific integrins and in some cases also requires a three-dimensional matrix (2, 12, 27, 29, 30). However, various integrins can serve to promote cell survival. For example, distinct  $\beta$ 1-integrins can mediate cell survival in mammary cells and chinese hamster ovary cells in vitro, whereas  $\alpha$ v $\beta$ 3 can mediate melanoma cell survival in three-dimensional collagen and survival of proliferating vascular cells in vivo (2, 12, 27, 29, 30). The integrin required for cell survival and the integrin-dependent survival signals might thus be cell type and condition specific.

While the use of receptor antagonists in vivo can suggest receptor function, we performed experiments to directly examine the effect of  $\alpha$ v $\beta$ 3 ligation on cultured human endothelial cells. In this case, antiintegrin antibodies were immobilized on a substrate facilitating their use as agonists of integrin function, thereby promoting adhesion of cells via clustering of a given integrin. This approach has been successfully used to examine integrin-specific signaling events in the absence of growth factors and other adhesion events, also demonstrating that distinct integrins mediate different signals (25, 41, 42).

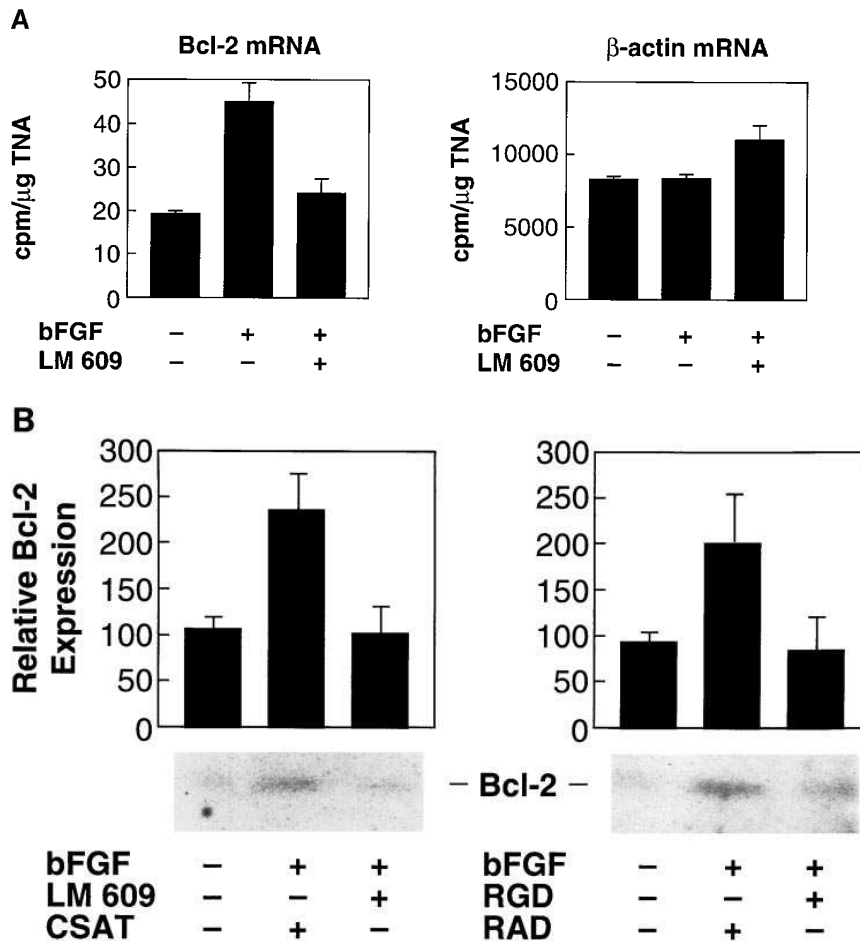
Inhibition of intercellular contact in a colon carcinoma cell line in vitro, mediated by an undefined  $\alpha$ v integrin, induces relocalization of p53 to the nucleus (39). However, it is unclear if



**Figure 6.** Integrin-dependent regulation of endothelial cell bcl-2 and bax protein levels. HUVECs were plated under serum free conditions on immobilized mAbs LM 609 (anti- $\alpha$ v $\beta$ 3), P4C10 (anti- $\beta$ 1), or PIF6 (anti- $\alpha$ v $\beta$ 3) or were denied attachment on a BSA-coated surface for 4 h. Cells were then collected and lysates were analyzed for Bcl-2 and bax protein levels by Western blotting as described in Methods. The bcl-2 and bax protein levels analyzed as above were then quantified by densitometry. The bars represent the relative ratio based upon quantities of bcl-2 and bax as measured by densitometry analysis of autoradiographs and do not represent an absolute molar ratio.

this translocation is associated with any change in p53 DNA binding activity. We here show that  $\alpha$ v $\beta$ 3-mediated endothelial cell adhesion selectively reduces p53 activity and p21<sup>WAF1/CIP1</sup> levels, since attachment via anti- $\beta$ 1 integrins or  $\alpha$ v $\beta$ 5 does not. Furthermore, the degree of cell spreading has been suggested as an important factor for endothelial cell survival (40). However, we demonstrate that attachment to either anti- $\alpha$ v $\beta$ 3 or anti- $\beta$ 1 resulted in equivalent cell spreading, yet these substrates differed in their ability to regulate p53 activity and p21<sup>WAF1/CIP1</sup> expression. Ligation of  $\alpha$ v $\beta$ 3 also resulted in a sharp increase in the bcl-2/bax ratio, consistent with the role of p53 as a transcriptional repressor of bcl-2 and an activator of bax (14–16). Based on the fact that bcl-2 potentiates cell survival by forming heterodimers with the death promoting molecule bax (31, 32), the increased bcl-2/bax ratio is expected to directly promote endothelial cell survival.

These studies provide a mechanism explaining how antagonists of  $\alpha$ v $\beta$ 3 can block angiogenesis and thereby cause regression of human tumor growth (2, 4). Experiments reported here demonstrate that  $\alpha$ v $\beta$ 3 ligation induces a distinct endothelial



**Figure 7.** Regulation of bcl-2 during chick CAM angiogenesis. (A) Angiogenesis was stimulated by bFGF in the chick CAM for 3 d in the presence or absence of mAb LM609 (anti- $\alpha\beta 3$ ) and bcl-2 mRNA and  $\beta$ -actin mRNA levels were measured by solution hybridization as described in Methods. Each bar represents the mean of three TNA preparations  $\pm$  SD. Each preparation was from four to five pooled CAM tissues. (B) Angiogenesis was stimulated as above in the presence of mAb LM 609 (anti- $\alpha\beta 3$ ) or mAb CSAT (anti- $\beta 1$ ) (left) or in the presence of cyclic RGD (anti- $\alpha\beta 3$ ) or RAD (control) peptides (right). CAM tissues were collected and cell lysates of four to five pooled CAMs were analyzed for bcl-2 protein levels by Western blotting under nonreducing conditions as described in Methods. The intensity of the band representing bcl-2 (indicated) was then quantified by densitometry (bars). Bars represent the mean values of three separate experiments  $\pm$  standard error.

cell survival signal in vitro and in vivo. Importantly, during bFGF- and tumor-induced angiogenesis, antagonists of this integrin block angiogenesis by promoting unscheduled apoptosis of newly growing blood vessels. Our findings provide a link between  $\alpha\beta 3$  ligation, p53 activity, expression of the cell cycle inhibitor p21<sup>WAF1/CIP1</sup>, and vascular cell survival. Thus, during angiogenesis, ligation of endothelial cell  $\alpha\beta 3$  is required for the suppression of apoptosis and of conflicting growth arrest signals, thereby facilitating the proliferation and maturation of new blood vessels.

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## References

1. Folkman, J., and Y. Shing. 1992. Angiogenesis. *J. Biol. Chem.* 267:10931-10934.
2. Brooks, P.C., A.M.P. Montgomery, M. Rosenfeld, R.A. Reisfeld, T. Hu,

- G. Klier, and D.A. Cheresch. 1994. Integrin  $\alpha\beta 3$  antagonists promote further tumor regression by inducing apoptosis of angiogenic bloodvessels. *Cell*. 79: 1157-1164.
3. Brooks, P.C., R.A.F. Clark, and D.A. Cheresch. 1994. Requirement of vascular integrin  $\alpha\beta 3$  for angiogenesis. *Science (Wash. DC)*. 264:569-571.
4. Brooks, P.C., S. Strömblad, R. Klemke, D. Visscher, F.H. Sarkar, and D.A. Cheresch. 1995. Antiintegrin  $\alpha\beta 3$  blocks human breast cancer growth and angiogenesis in human skin. *J. Clin. Invest.* 96:1815-1822.
5. Ruoslahti, E., and J.C. Reed. 1994. Anchorage dependence, integrins, and apoptosis. *Cell*. 77:477-478.
6. Guadagno, T.M., M. Ohtsubo, J.M. Roberts, and R.K. Assoian. 1993. A link between cyclin A expression and adhesion-dependent cell cycle progression. *Science (Wash. DC)*. 262:1572-1575.
7. Wu, X., and A.J. Levine. 1994. p53 and E2F-1 cooperate to mediate apoptosis. *Proc. Natl. Acad. Sci. USA*. 91:3602-3606.
8. Almasan, A., Y. Yin, R.E. Kelly, E.Y.-H.P. Lee, A. Bradley, W. Li, J.R. Bertino, and G.M. Wahl. 1995. Deficiency of retinoblastoma protein leads to inappropriate S-phase entry, activation of E2F-responsive genes, and apoptosis. *Proc. Natl. Acad. Sci. USA*. 92:5436-5440.
9. Symonds, H., L. Krali, L. Remington, M. Saenz-Robles, S. Lowe, T. Jacks, and T. van Dyke. 1994. p53-dependent apoptosis suppresses tumor growth and progression in vivo. *Cell*. 78:703-711.
10. Yonish-Rouach, E., D. Grunwald, S. Wilder, A. Kimichi, E. May, J.-J. Lawrence, P. May, and M. Oren. p53-mediated cell death: relationship to cell cycle control. *Mol. Cell. Biol.* 13:1415-1423.
11. Meikrantz, W., and R. Schlegel. 1995. Apoptosis and the cell cycle. *J. Cell. Biochem.* 58:160-174.
12. Boudreau, N., Z. Werb, and M.J. Bissell. 1996. Suppression of apoptosis by basement membrane requires three-dimensional tissue organization and withdrawal from the cell cycle. *Proc. Natl. Acad. Sci. USA*. 93:3509-3513.
13. El-Deiry, W., J.W. Harper, P.M. O'Connor, V.E. Velculescu, C.E. Cattan, J. Jackman, J.A. Pietenpol, M. Burrell, D.E. Hill, Y. Wang, et al. 1994. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res.* 54:1169-1174.
14. Miyashita, T., S. Krajewski, M. Krajewski, H.G. Wang, H.K. Lin, D.A. Liebermann, B. Hoffman, and J.C. Reed. 1994. Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. *Oncogene*. 9:1799-1805.
15. Miyashita, T.S., M. Harigari, M. Hanada, and J.C. Reed. 1994. Identifi-

cation of a p53-dependent negative response element in the bcl-2 gene. *Cancer Res.* 54:3131–3135.

16. Miyashita, T.S., and J.C. Reed. 1995. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell.* 80:293–299.

17. Durnam, D.M., and R.P. Palmiter. 1983. A practical approach for quantitating specific mRNA by solution hybridization. *Anal. Biochem.* 131:385–393.

18. Strömblad, S., and G. Andersson. 1993. The coupling between the transforming growth factor  $\alpha$  and the epidermal growth factor receptor during rat liver regeneration. *Exp. Cell Res.* 204:321–328.

19. Cazals-Hatem, D.L., D.C. Louise, S. Tanaka, and J.C. Reed. 1992. Molecular cloning and DNA sequence analysis of cDNA encoding chicken homologue of the bcl-2 oncoprotein. *Biochim. Biophys. Acta.* 1132:109–113.

20. Cleveland, D.W., M.A. Lobata, R.J. McDonald, N.J. Cowan, W.J. Rutter, and M.W. Kischner. 1980. Number and evolutionary conservation of  $\alpha$  and  $\beta$ -tubulin and cytoplasmic  $\beta$ - and  $\gamma$ -actin genes using specific cloned cDNA. *Cell.* 20:97–105.

21. Schreiber, E., P. Matthias, M.M. Müller, and W. Schaffner. 1989. Rapid detection of octamer binding proteins with mini extracts, prepared from a small number of cells. *Nucl. Acids Res.* 17:6419.

22. Harper, J.W., G.R. Adami, N. Wei, K. Keyomarsi, and S.J. Elledge. 1993. The p21 cdk interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell.* 75:805–816.

23. El-Deiry, W., T. Tokino, V.E. Velculescu, D.B. Levy, R. Parsons, J.M. Trent, D. Lin, W.E. Mercer, K.W. Kinzler, and B. Vogelstein. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell.* 75:817–825.

24. Luo, Y., J. Hurwitz, and J. Massague. 1995. Cell-cycle inhibition by independent CDK and PCNA binding domains in p21Cip1. *Nature (Lond.).* 375:159–161.

25. Leavesley, D.I., M.A. Schwartz, M. Rosenfeld, and D.A. Cheresh. 1993. Integrin  $\beta$ 1- and  $\beta$ 3-mediated endothelial cell migration is triggered through distinct signal mechanisms. *J. Cell Biol.* 121:163–170.

26. Meredith, J.E., B. Fazeli, and M.A. Schwartz. 1993. The extracellular matrix as a cell survival factor. *Mol. Biol. Cell.* 4:953–961.

27. Montgomery, A.M.P., R.A. Reisfeld, and D.A. Cheresh. 1994. Integrin  $\alpha$ v $\beta$ 3 rescues melanoma cells from apoptosis in three-dimensional dermal collagen. *Proc. Natl. Acad. Sci. USA.* 91:8856–8860.

28. Frisch, S.M., and H. Francis. 1994. Disruption of epithelial cell-matrix interactions induces apoptosis. *J. Cell Biol.* 124:619–626.

29. Boudreau, N., C.J. Simpson, Z. Werb, and M.J. Bissel. 1995. Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix.

*Science (Wash. DC).* 267:891–893.

30. Zhang, Z., K. Vouri, J.C. Reed, and E. Rouslahti. 1995. The  $\alpha$ 5 $\beta$ 1 integrin supports survival of cells on fibronectin and up-regulates bcl-2 expression. *Proc. Natl. Acad. Sci. USA.* 92:6161–6165.

31. Oltvai, Z.N., C.L. Millman, and S.J. Korsmeyer. 1993. Bcl-2 heterodimerizes in vivo with a conserved homolog, bax, that accelerates programmed cell death. *Cell.* 74:609–619.

32. Yin, X.-M., Z.N. Oltvai, S.J. Korsmeyer. 1994. BH1 and BH2 domains of bcl-2 are required for inhibition of apoptosis and heterodimerization with bax. *Nature (Lond.).* 369:321–323.

33. Fang, F., G. Orend, N. Watanabe, T. Hunter, and E. Ruoslahti. 1996. Dependence of cyclin E-cdk2 kinase activity on cell anchorage. *Science (Wash. DC).* 271:499–502.

34. Drake, C.J., D.A. Cheresh, C.D. Little. 1995. An antagonist of integrin  $\alpha$ v $\beta$ 3 prevents maturation of blood vessels during embryonic neovascularization. *J. Cell Sci.* 108:2655–2661.

35. Friedlander, M., P.C. Brooks, R.W. Shaffer, C.M. Kincaid, J.A. Varner, and D.A. Cheresh. 1995. Definition of two angiogenic pathways by distinct  $\alpha$ v integrins. *Science (Wash. DC).* 270:1500–1502.

36. Cheresh, D.A. 1987. Human endothelial cells synthesize and express an Arg-Gly-Asp-directed receptor involved in attachment to fibrinogen and von Willebrand factor. *Proc. Natl. Acad. Sci. USA.* 84:6471–6475.

37. Felding-Habermann, B., and D.A. Cheresh. 1993. Vitronectin and its receptors. *Curr. Opin. Cell Biol.* 5:864–868.

38. Brooks, P.C., S. Strömblad, L.C. Sanders, T.L. von Schalscha, R.T. Aimes, W.G. Stetler-Stevenson, J.P. Quigley, and D.A. Cheresh. 1996. Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin  $\alpha$ v $\beta$ 3. *Cell.* 85:683–693.

39. Bates, R.C., A. Buret, D. van Helden, M.A. Horton, and G.F. Burns. 1994. Apoptosis induced by inhibition of intercellular contact. *J. Cell Biol.* 125:403–415.

40. Re, F., A. Zanetti, M. Sironi, N. Polentarutti, L. Lanfranccone, E. Dejana, and F. Colotta. 1994. Inhibition of anchorage-dependent cell spreading triggers apoptosis in cultured human endothelial cells. *J. Cell Biol.* 127:537–546.

41. Schwartz, M.A., and K. Denninghoff. 1994.  $\alpha$ v integrins mediate the rise in intracellular calcium in endothelial cells on fibronectin even though they play a minor role in adhesion. *J. Biol. Chem.* 269:11133–11137.

42. Miyamoto, S., S.K. Akiyama, and K.M. Yamada. 1995. Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. *Science (Wash. DC).* 267:883–885.