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In an adhesion dependent human gastric adenocarcinoma cell line, integrin ligation without adhesion rescues from anoikis but is not sufficient for cell cycle progression

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Abstract

STAD cells are the adherent parental apoptotic line from which two sublines were cloned that differed in their response to suspended culturing conditions, one clone STAD.APO is apoptotic and the other STAD.ARR goes into cell cycle arrest. Using this system we have found that the addition of soluble collagen can rescue STAD and STAD.APO cells from anoikis, and it can also affect STAD.ARR cells by overcoming the suspension induced cell cycle arrest. In contrast, when cells were cultured with a soluble anti-ß1 integrin mAb 33B6, the apoptotic clones again were rescued from anoikis, but the cell cycle arresting clone remained quiescent. This result was somewhat surprising as it is generally accepted that cytoskeletal rearrangements that accompany integrin mediated adhesion and cell shape changes are required for the abrogation of anoikis, and it was unexpected that differences in the mechanism used for integrin triggering would yield variable results on growth regulation. This observation led us to further examine whether the addition of a monovalent anti-ß1 integrin agent could produce similar results as intact mAb. Therefore we employed Fab fragments of 33B6 in our culturing assay and found that indeed monovalent binding was capable of saving STAD and STAD.APO cells from anoikis but did not have an effect on STAD.ARR cells. Therefore in this study we have observed that integrin mediated dependent survival can occur by mere ligation of the ß1 integrin subunit, but that cell cycle arrest due to suspended conditions can not. Thus integrins can play differential roles in cell fate decisions and mediate these effects by different mechanisms. *Cell Death and Differentiation* (2001) **8,** 665–678.

Keywords: integrins; anoikis; gastric adenocarcinoma; cell cycle arrest; adhesion

Abbreviations: STAD, stomach tumor adhesion dependent; STAD.APO, stomach tumor adhesion dependent apoptotic; STAD.ARR, stomach tumor adhesion dependent cell cycle arresting; FN, fibronectin; Coll, collagen; BSA, bovine serum albumin; PI, propidium iodide; ECM, extracellular matrix; mAb, monoclonal antibody; ST2, stomach tumor-2 cell line; poly-HEMA, poly -2-hydroxyethyl methacrylate; MFI, mean fluorescence intensity; FAK, focal adhesion kinase; FBS, fetal bovine serum; PBS, phosphate buffered saline; ITS, insulin, transferrin and selenium; RT, room temperature; EDTA, ethylenediaminetetraacetic acid; FITC, fluorescein isothiocyanate

Introduction

Integrins are heterodimeric transmembrane cell surface receptors composed of an α subunit and a β subunit that mediate cell adhesion to the extracellular matrix as well as to other cells. In humans there are 16 α subunits and eight β subunits that combine to form 22 integrins with various ligand specificities.[1] Integrin mediated adhesion is responsible for transducing signals to rearrange the actin cytoskeleton to affect morphology, as well as signaling to the nucleus to control cellular proliferation, differentiation and apoptosis. Adhesion via integrins has been implicated in the outcome of cell fate decisions, such that cellular function and life or death responses to integrin ligation or the lack of integrin ligation, can determine the most basic of cellular behaviors.[2] Due to the importance of adhesion for the control of cell physiology, the quest to understand downstream events of integrin ligation has become an area of active investigation with regards to the problem of cancer.

One hallmark of oncogenic transformation is the loss of adhesion dependent proliferation. Since this adhesion is largely an integrin mediated event, the role that integrins play in the control of cell cycle progression may prove to be an important mechanism of carcinogenesis, yet it is poorly understood. Non-transformed cells can exhibit an adhesion requirement for cell survival and when denied adherence will then undergo apoptosis (anoikis), in response to an inappropriate local environment.[3,4] When the checkpoint of anoikis is lost, cells are able to either proliferate without regulatory constraints of adhesion or enter into cell cycle arrest until a suitable environment is available to them. Cells that continue to proliferate without adhesion may then lead to malignant tumor growth, and metastasis. Cells that become quiescent in response to a lack of adhesion are able to travel to distant sites and cause metastatic lesions. This scenario may present a model for metastasis and tumor dormancy, and the role that integrins may play in these events.

Oncogenesis and metastasis are complex and difficult events to understand and to investigate however it has

recently been appreciated that cellular proliferation is under the control of both soluble mitogenic growth factors and integrin-mediated adhesion. Hence, we find that cancer cells comprise a heterogenous population that are either anchorage independent, growth factor independent or independent for both growth factors and anchorage for proliferation. The role integrins play in cancer and metastasis can be examined in the context of the multi-step paradigm of oncogenesis.[2,5] At each step in the progression towards cancer and metastasis a level of control over cellular behavior is lost. Once the cell becomes anchorage independent by some unknown mechanism that may or may not be related to integrin expression and/or function, the transformed cell can detach from the extracellular matrix (ECM) and proliferate freely in the periphery. An alternative scenario is that the transformed cell merely enters cell cycle arrest upon detachment from the ECM and does not proliferate but is free to circulate in the periphery until a suitable environment is encountered where it can adhere and reenter the cell cycle resulting in a metastatic lesion. Additionally, since most chemotherapeutic agents target cycling cells, the quiescent cell can evade traditional therapies perhaps for an extended length of time and create a secondary tumor. This model attributes an important role to integrins in oncogenesis and metastasis.

The metastatic process requires, both the down regulation and up regulation of cell surface integrin expression or function, at different stages in the progression of carcinoma. While the majority of tumors show a decrease or defect in integrin expression or function, there is also the case where increased integrin expression confers the malignant phenotype.[2] The integrin $\alpha5\beta1$ has been shown in MG63 osteosarcoma cells and K562 erythroleukemia to be an important regulator for the tumorigenicity of these cell lines. Subpopulations of both cell lines were selected for their ability to bind fibronectin (FN). Once analyzed it was found that these adhesive populations overexpressed $\alpha5\beta1$ compared to parentals and demonstrated reduced anchorage independence for proliferation and reduced tumorigenicity.[6] In Chinese hamster Ovary cells that do not normally express $\alpha5\beta1$, it was shown that transfected $\alpha5\beta1$ and its subsequent overexpression prevented tumorigenicity and loss of anchorage independence. In human breast carcinoma it was shown by Zutter *et al*. that the forced expression of the $\alpha2\beta1$ integrin reversed the malignant phenotype and restored normal morphology and differentiation.[7] Yet, the reverse condition exists, where upregulation of integrin expression was associated with the transformed phenotype. This has been demonstrated in several studies using melanoma cells. Albelda *et al*. showed that $\alpha v\beta3$ integrin expression associated with the metastatic ability of melanoma cells, when compared to benign controls.[8] In another study, cells were selected for the loss of the αv subunit and were shown to have reduced tumorigenicity and proliferation which was reversed when αv was re-expressed in these cells.[6] Hence there can be different ramifications of the integrin expression level that may be integrin specific, cell type specific or associated with cell state specificities such as differentiation state or cell cycle phase.

The focus of this investigation of integrin function is to determine the relationship between integrins and their effect on cell fate. In our laboratory we have isolated a human metastatic gastric adenocarcinoma cell line, ST2, that when denied adherence much of the population was shown to enter cell cycle arrest.[9,10] This event was mediated by the integrin $\alpha2\beta1$ which is the receptor for both collagen and laminin. However, it was discovered that adhesion on collagen and not laminin was responsible for adhesion dependent proliferation. When ST2 cells were incubated with anti-$\alpha2$ and anti-$\beta1$ monoclonal antibodies (mAb) and then plated on collagen ST2 cells were prohibited from binding to the substrate and demonstrated rounded morphology and cell cycle arrest.[9] Additionally, it was observed that while some of the ST2 cells in suspension arrested at the G0/G1 restriction point, the remainder of the cells were dying by apoptosis. This suggested that there existed subpopulations of ST2 cells that did not respond to the loss of adhesion in the same manner. Thus to further explore integrin mediated cellular behavior and to examine the mechanisms of integrin mediated cell fate determination, we undertook to separate these two populations to examine the role of integrins on the differing integrin mediated phenotypes. This is an intriguing model as it afforded us with the opportunity of determining the relationship between two genetically related but phenotypically different populations from the same disease site.

To that end we undertook a series of experiments to understand how integrins may be regulating these differing phenotypes. The addition of an anti-ß1 integrin mAb 33B6 to cells in suspended conditions revealed that this treatment could save apoptotic cell lines from death, but had no effect on the cell cycle arrested line. Soluble collagen was able to rescue from apoptosis in a similar manner as mAb 33B6. Additionally, monovalent 33B6 could also save from apoptosis, showing that neither cell shape change nor integrin clustering due to extracellular cross-linking was required for the prevention of apoptosis. This suggests that mere ligation of the integrin ß1, and not true adhesion and the cytoskeleton rearrangements involved in that event, can alter a cells response to a death inducing situation. Interestingly, this same ligation is not enough to push these cells through the cell cycle to proliferation.

Results

The STAD parental population contains at least two clonetypes

It has long been established that tumors are heterogeneous,[11,12] containing cells with different phenotypes. In our studies with ST2 gastric adenocarcinoma cells we noted during cell cycle analyses that two distinct populations appeared to coexist when denied adherence, one that entered into cell cycle arrest and the other entered apoptosis. To investigate the relationship between the two phenotypic varying populations of ST2 cells, it was first necessary to determine whether or not this effect was due to the possibility that the apoptotic cell subpopulation was in a

cell cycle stage that was unable to arrest at the time of transfer to suspension conditions and vice versa. This variance in the cycle may or may not include cell cycle specific states or altered growth kinetics. If cell state was the explanation for a proportion of the cells to apoptose or arrest in response to loss of adhesion, it would seem reasonable that an effort to clone out the two different phenotypic subpopulations would fail. Only if the cells were distinctly and stably different, would it be possible to arrive at two phenotypically divergent populations.

The cloning strategy was straight forward, we merely performed serial dilutions of the ST2 parental cell line (from this point on referred to as STAD cells) until we arrived at single cell clones. These were grown under standard conditions. Once these populations were expanded, they were assayed by propidium iodide staining for cell cycle distribution including the sub G0/G1 population, under adhered and suspended conditions. From this information it was determined which clones were predominantly apoptotic and which clones were predominantly entering cell cycle arrest when denied adherence, when cultured in complete media on poly-HEMA for 24 h. The effort to clone out both the apoptotic phenotype, STAD.APO and the arresting phenotype, STAD.ARR was successful as determined by cell cycle analysis as performed by flow cytometry (Figure 1). When denied adherence on poly-HEMA, the parental STAD cell line shows a substantial sub G1 peak indicative of apoptosis resulting in 55.4% death. The apoptotic clone STAD.APO shows 50.0% apoptosis in the sub G1 peak while the cell cycle arrest clone STAD.ARR only shows 15.9% apoptosis. Thus, the parental population indeed was heterogeneous and contained at least two stable distinct clonetypes. Furthermore, since no differences were found by karyotype analyses, there appears to be no major gross genetic alterations between the different subclones (data not shown). With this model whereby cells have the same genetic background, no major chromosomal aberrations from each other, and obtained from the same disease site, we can now address the issue of adhesion dependency for both survival/death and growth/arrest separately in a system that provides us with few variables between cell types.

Cell surface integrin expression analysis

To determine whether or not these subclones' differing phenotypes in regard to adhesion dependency were due to expression levels of cell surface receptors we employed a panel of integrin specific antibodies and performed flow cytometry on the cell lines. Here the cells were harvested directly from tissue culture flasks and stained as described in Materials and Methods. As shown in Table 1, the major integrin subunits detected on a high percentage of cells were $\beta1$, $\alpha2$, and $\alpha3$ indicating that the major integrins on these cells are $\alpha2\beta1$ and $\alpha3\beta1$. Moderate expression of $\alpha6$ and $\beta4$ was also detected. By examining both the percent positive expression and also the mean fluorescence intensity (MFI) between the three populations, in general very little variability was detected between the parental STAD, the apoptotic STAD.APO and the cell cycle arrest clone STAD.ARR. The lone exception was the higher expression of $\alpha3$ on the STAD.APO population. Since the STAD.APO line and the STAD line have similar phenotypes, and STAD and STAD.ARR have similar levels of $\alpha3$, it does not appear that $\alpha3$ levels are indicative of the apoptotic phenotype. Therefore, the different behaviors of STAD.APO and STAD.ARR is not clearly attributed to expression levels of these integrins.

Integrin function as determined by cell spreading on ECM

Many adherent cells secrete extracellular matrix (ECM) components such as fibronectin, laminin and collagen, and it is this secreted ECM that coats the tissue culture plastic to which the cells adhere and spread via integrin ligation. Previously, it was shown that the original cell from which these sublines were derived, ST2, preferred collagen as its substrate for adhesion and spreading through the integrin $\alpha2\beta1$.[9] Since the sublines STAD, STAD.APO and STAD.ARR were now exhibiting different phenotypes it was important to determine whether different integrins and ECM components were likely responsible for their adhesion and spreading.

Cells were seeded onto either purified collagen, laminin or fibronectin in the presence or absence of blocking anti-integrin antibodies. By observing the degree and kinetics by which the cells would spread, or not, in response to matrix and antibody we were able to determine which integrins were mediating adhesion and spreading for each of the cell lines. As shown in Figure 2, each cell line spread extensively and rapidly on collagen within 1 h of being plated and incubated at 37°C. On fibronectin or laminin, no spreading was observed at 1 h, and even after 2 h the cells exhibited minimal spreading (data not shown). Since the cells predominantly utilized collagen for spreading, this narrows the possible integrin candidates for spreading on matrix components to $\alpha2\beta1$ and $\alpha3\beta1$.[13]

$\beta1$ integrin is the predominant integrin subunit involved in adhesion to ECM components and we show here that indeed incubation with an adhesion blocking $\beta1$ mAb, 33B6 effectively inhibits each cell lines' ability to adhere and spread on each ECM component (Figure 2). Therefore, we can conclude that $\beta1$ is indeed an important mediator of adhesion in these cell lines. To determine the α subunit involvement, cells from each subline were seeded on collagen in the presence of blocking $\alpha2$ and $\alpha3$ antibodies. As seen in Figure 2 only $\alpha2$ was effective in blocking spreading on collagen for each subline. Thus, by these series of experiments we see that $\alpha2\beta1$ is the integrin mediating spreading for STAD, STAD.APO and STAD.ARR and that their phenotypes are not due to differential integrin function. The conservation of integrin expression and usage in these related sublines may indicate that even under suspended conditions, different integrins are not mediating the behavioral variation.

Soluble collagen rescues STAD and STAD.APO from apoptosis and can effect cell cycle progression of STAD.ARR cells

The spreading experiments on ECM components suggests that collagen can serve as the preferred substrate for these

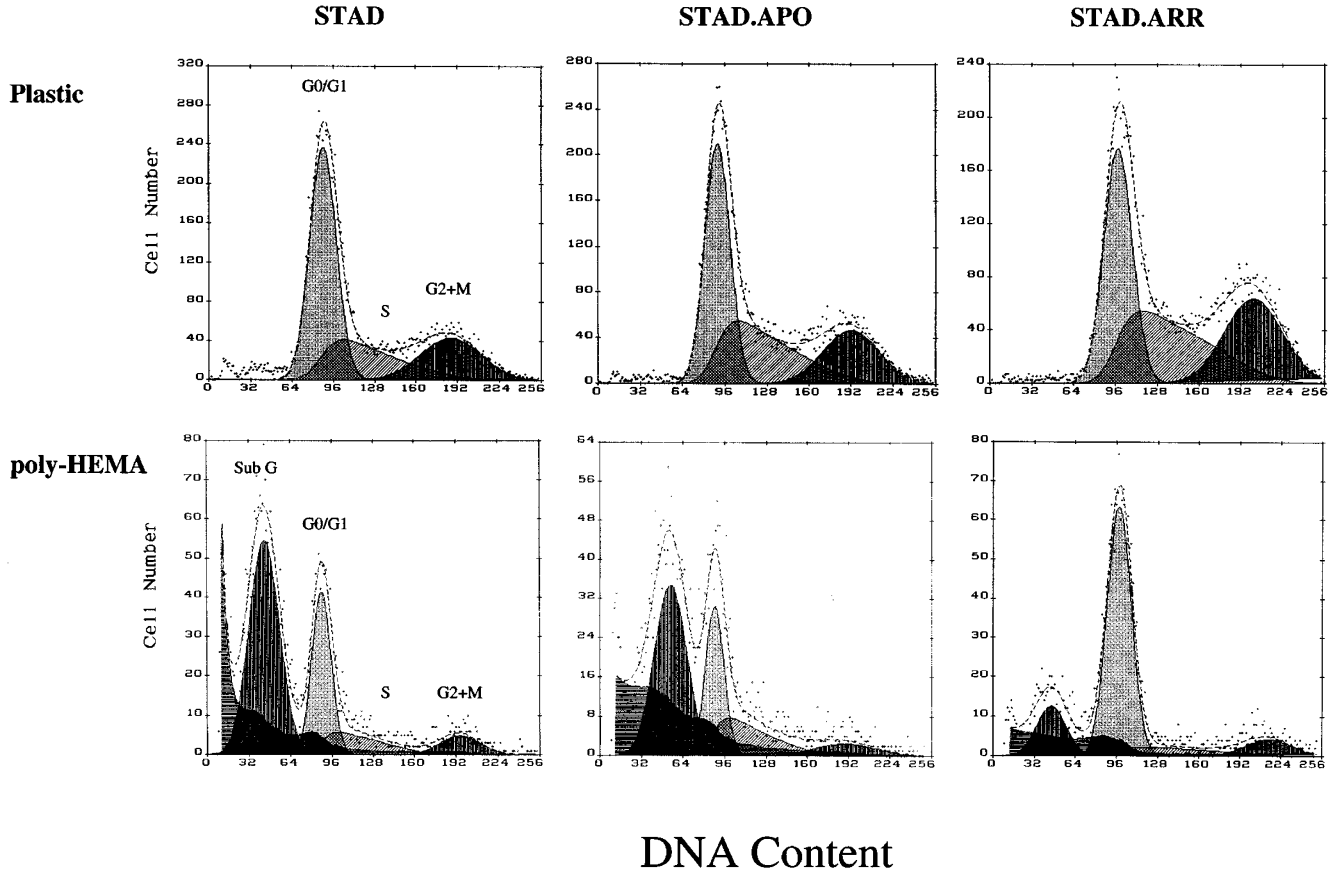


Figure 1 STAD and STAD.APO cells undergo apoptosis when denied adherence, while STAD.ARR enters cell cycle arrest. 10^5 cells per ml of complete media were cultured for 24 h either on untreated tissue culture plastic or on poly-HEMA coated tissue culture plastic in 6 well plates. Cells were harvested by standard methods and fixed with ice cold 70% ethanol overnight at 4°C. Cells were stained with propidium iodide and analyzed by flow cytometry as described

Table 1 Cell surface integrin profiles

	STAD.APO		STAD.APR		STAD	
mAb	**%+**	**MFI**	**%+**	**MFI**	**%+**	**MFI**
β1	100	551.0	100	430.9	99.7	291.1
β4	38.4	54.66	29.4	48.45	27	53.49
α1	3.6	148.3	3.4	51.31	7.6	64.67
α2	100	552.6	99.9	425.2	100	362.5
α3	98	184.9	96.3	36.5	97	58.31
α4	8.9	85.54	5.8	67.17	8.3	64.78
α5	11.1	70.85	2.5	53.89	7.8	63.52
α6	30.3	46.2	26.1	46.25	21.7	49.79
αv	4.4	81.53	3.8	57.49	7.6	60.39

3×10^5 cells from each cell line were stained with various anti-integrin subunits in the presence of 1% goat serum for 30 min on ice. After incubation samples were washed with PBS and the secondary antibody, goat anti-mouse conjugated with FITC was added at a final concentration of 1:1000 and incubated on ice for 30 min. After a final wash with PBS samples were analyzed by flow cytometry. Results are presented in percent positive (%+) and mean fluorescence intensity (MFI)

cells. To that end we asked whether soluble collagen has the ability to save the apoptotic clones from suspension induced death. A titration of soluble collagen at 0.1, 1.0 and 10 μg/ml was used to test if collagen can decrease apoptotic death in STAD and STAD.APO cells in complete media and incubated for 24 h either on untreated tissue culture plastic or on poly-HEMA coated wells. As shown in Figure 3, results are reported in percentage decrease in apoptosis by comparing percentage of cells in sub G1 on plastic *versus* that on poly-HEMA in the following relationship 1- [(poly-HEMA - control)/ poly-HEMA with mAb - control with mAb)]. STAD cells showed a 51% decrease in death at the highest concentration of soluble collagen (Figure 3A). STAD.APO demonstrated a 73% decrease in death at 10 μg/ml of collagen (Figure 3B). With the STAD.ARR cells (Figure 3C), the few cells that do die when cultured on poly-HEMA were saved by soluble collagen.

Anti-β1 integrin mAb rescues STAD and STAD.APO from apoptosis but has no effect on STAD.ARR

Since the lack of adhesion caused cells to undergo apoptosis, the integrin ß1 was regulating that adhesion, and soluble collagen could inhibit apoptosis, we tested whether soluble 33B6 may also be able to rescue cells from anoikis. To this end we performed a series of experiments to test this idea. First, a titration of 33B6 ascites was done at 1:10 through 1:100 000 dilution in complete media and cells were seeded at a density of 10^5 cells per ml on either uncoated tissue culture plastic or on poly-HEMA coated wells

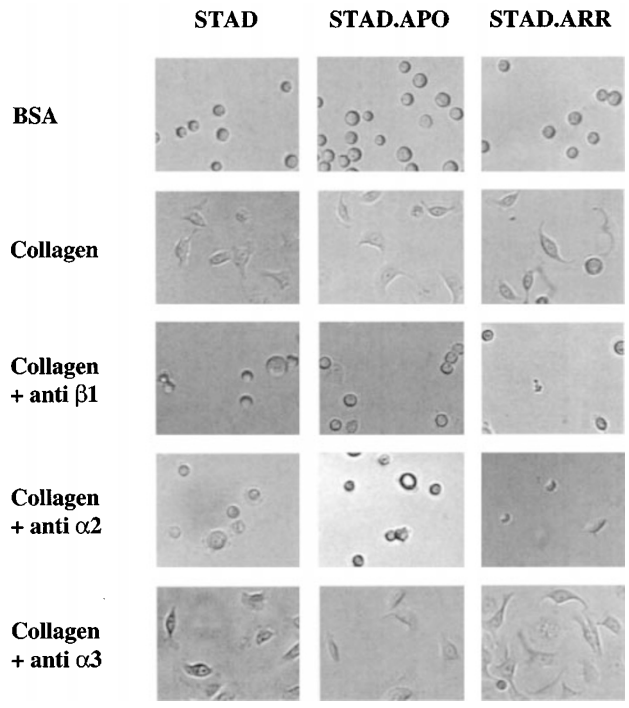


Figure 2 Antibodies to α2 and β1 integrin subunits inhibit cell spreading on collagen. Collagen was coated onto tissue culture plastic wells as described in Materials and Methods. STAD, STAD.APO and STAD.ARR cells were plated on BSA or collagen and incubated in the presence of blocking mAbs to integrin β1, α2 and α3 subunits where indicated

for 24 h at 37°C. Cells were harvested, stained with PI and analyzed by flow cytometry as described in Materials and Methods. As shown in Figure 4A, at the highest concentration of 33B6 a 44.3% decrease in the cells undergoing apoptosis due to loss of adhesion in the STAD cell line was found. In the STAD.APO cell line (Figure 4B), a similar effect of 33.7% reduction in death with a 1 : 10 dilution of 33B6 ascites was seen. These titration curves show that the effect is due to 33B6 and most optimally at either 1 : 10 or 1 : 100 dilution of ascites. To insure that the effect was specific to β1 integrin ligation we repeated these experiments with purified 33B6 (10 μg/ml) and found similar results (data not shown). As shown in Figure 4C, the results from a 33B6 ascites titration with the STAD.ARR cells may also indicate an antibody mediated decrease in apoptosis. However, this may be a non-specific response and is difficult to measure because such a small proportion of the cells are dying by apoptosis. These results indicate that the rescue from anoikis can occur in an adhesion free environment if integrin receptors and any associated cytoskeletal molecules are rearranged by crosslinking.

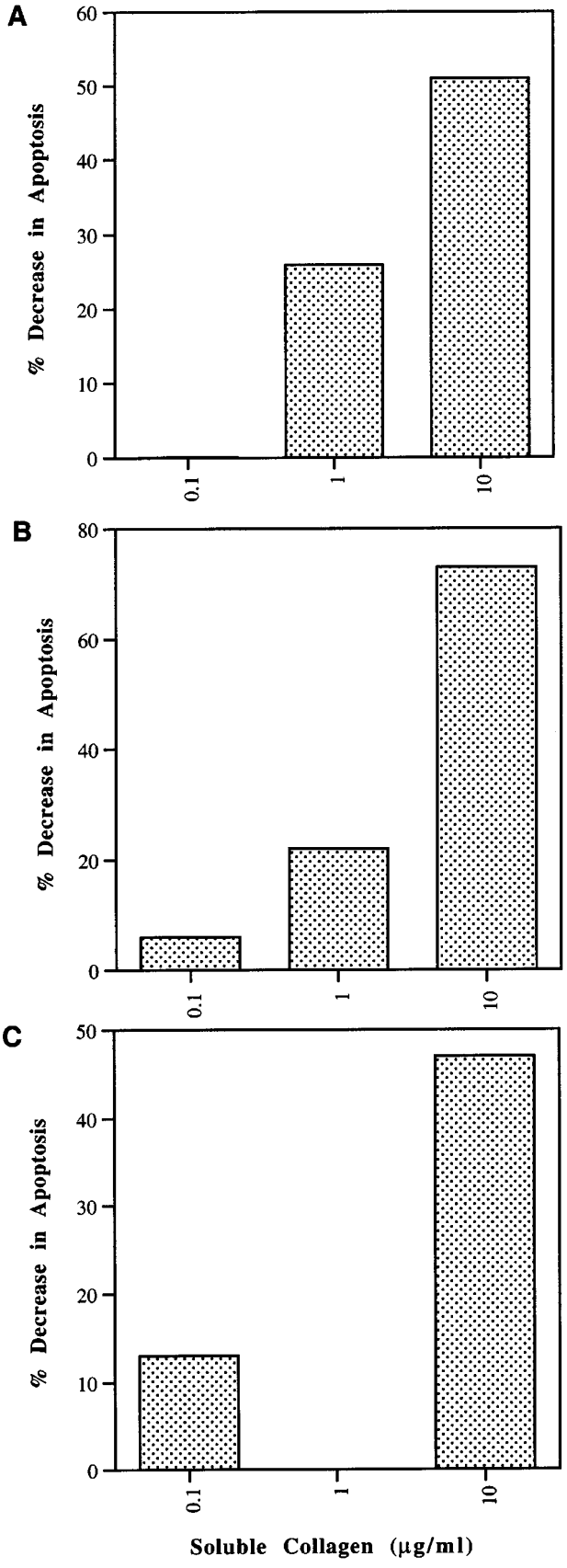
A different β1 integrin mAb 18D3 does not rescue from apoptosis

18D3 is an anti-β1 integrin specific mAb maintained in our laboratory that has been shown to bind to a distinct epitope from mAb 33B6 and has negligible effects on cell adhesion. This mAb was employed to determine whether β1 integrin crosslinking in general was sufficient to inhibit apoptosis. A 1 : 100 dilution of 18D3 ascites was mixed with the cells just prior to seeding onto uncoated tissue culture wells or on poly-HEMA coated tissue culture wells. After a 24 h incubation at 37°C the cells were harvested and stained with PI and analyzed by flow cytometry for apoptotic cells. As shown in Figure 5A the addition of 18D3 had a very slight effect on STAD cells. Without mAb, STAD cells showed 54.5% apoptosis while with 18D3 treatment 48.1%. In Figure 5B the STAD.APO cells showed 33.6% death without treatment but with the addition of 18D3 we see 36.1% death. In Figure 5C the addition of 18D3 to STAD.ARR cells resulted in 7.1% death compared to the no treatment control that resulted in 13.9% death. Therefore, not mere ligation and crosslinking of β1 but the proper epitope must be engaged to rescue from apoptosis.

Time course of 33B6 addition and kinetics of 33B6 effect

To further investigate the properties of the 33B6 effect on the apoptotic cell lines two different kinetic studies were performed. The first, shown in Figure 6A, examines at what point in a 24 h assay that the addition of 1 : 100 dilution of 33B6 ascites can still rescue STAD cells from apoptosis when cultured on poly-HEMA coated wells. As expected the earlier that 33B6 was added to the cells and hence the longer the incubation with 33B6 the more profound the effect. The time 0 condition had 33B6 added at the beginning of the assay and therefore represents 24 h of incubation with 33B6 and shows a 35% decrease in death. At the addition after 6 h on poly-HEMA, STAD cells show a 27% decrease in death, and after 12 h the addition of 33B6 had a much reduced effect compared with the conditions where 33B6 is added earlier in the assay.

The second study asked what duration of 33B6 incubation was required to produce the survival effect on poly-HEMA. Figure 6B summarizes the results of plating cells alone or cells mixed with 1 : 100 33B6 ascites on poly-HEMA coated wells or on uncoated tissue plastic, and then harvested at the times indicated. Without 33B6 incubation, the percentage of STAD cells becoming apoptotic steadily rose with time and at approximately 24 h dramatically increased to 59.5% (Figure 6B, top panel). After 6 h, STAD cells show no effect from 33B6 addition, however as early as 14 h a slight 33B6 protection effect is seen, and between 18 and 24 h the amount of protection continued to increase. Similarly, the STAD.APO subline was protected by 33B6 (Figure 6B, middle panel) at all time points but most significantly at the later time points, showing 13.3% death after 24 h incubation with 33B6 compared to the control without 33B6 that showed 35.2% death. In contrast, the effect of 33B6 on the overall survival of STAD.ARR was inconsequential due to the fact that few of these cells are dying (Figure 6B, bottom panel). Of the cells that do undergo apoptotic death there seemed to be some amount of rescuing at the latest time points, for the incubation with 33B6 for 21 and 24 h shows apoptosis at 5.5% and 0.1% respectively compared with the no 33B6 control which shows 10.6% and 12% death.



33B6 Fab fragments rescues STAD and STAD.APO from apoptosis but has no effect on STAD.ARR

To investigate whether 33B6's ability to rescue from anoikis was due to bivalent receptor crosslinking, or if the 33B6 effect was due to mere ligation of the proper β1 integrin epitope, we generated Fab fragments from purified 33B6 and employed them in our culturing assay. Figure 7 shows the results of those experiments. A titration of 33B6 Fab at 25, 50 and 100 μg/ml was used to evaluate the efficacy of 33B6 Fab in this system. STAD cells had decreased apoptosis by 27% at the lowest concentration of 33B6 Fab compared to STAD cells cultured without 33B6 Fab. At 100 μg/ml of 33B6 Fab STAD cells demonstrated a 70% decrease in apoptosis due to lack of adhesion. STAD.APO cells were also saved from suspension induced apoptosis due to treatment with 33B6 Fab. At 25 μg/ml 33B6 Fab, STAD.APO cells were saved at 11% but at 100 μg/ml these cells were saved by 50% by treatment with 33B6 Fab. STAD.ARR cells, by contrast, did not show a dose response to 33B6 Fab treatment. STAD.ARR cells showed a decrease in apoptosis of 20% at 25 μg/ml and at 100 μg/ml 22%. Thus, 33B6 Fab can also save apoptotic cells lines from suspension induced death similarily to intact 33B6 mAb treatment, and thus the actions associated with the extracellular crosslinking of receptor are not required for integrin dependent cell survival.

Suspension induced cell cycle arrest in STAD.ARR cells can be overcome with treatment with soluble collagen, but not 33B6, 18D3 or 33B6 Fab fragments

As previously shown by our laboratory[9] immobilized collagen was able to re-initiate the cell cycle in suspension induced cell cycle arrested cells. To examine if soluble collagen would be able to prevent cell cycle arrest due to lack of adhesion we performed a titration with STAD.ARR cells in our standard culturing assay. Cells were incubated with collagen at the concentrations indicated for 24 h on poly-HEMA in complete media and then harvested, fixed and stained with PI for cell cycle analysis by flow cytometry. As shown in Table 2 soluble collagen had an effect on the progression of the cell cycle for STAD.ARR cells. The greatest effect was seen at 10 μg/ml soluble collagen STAD.ARR cells on poly-HEMA showed 33.9% G1, 25.7% G2 and 40.4% S, compared to no treatment poly-HEMA that showed 74.5% G1, 15.3% G2 and 10.1% S.

Treatment with anti-β1 mAbs that were successful in preventing anoikis proved not to be sufficient for the prevention of cell cycle arrest in STAD.ARR cells as seen

Figure 3 Soluble collagen rescues apoptotic cells from apoptosis. A titration of soluble collagen from 0.1, 1.0 and 10 μg/ml was used in our standard culturing assay. 3×10^5 cells were incubated in complete media without collagen or with collagen at the concentrations indicated for 24 h at 37 C, then cells were harvested by standard methods and fixed with ice cold 70% ethanol overnight at 4°C. Cells were stained with propidium iodide and analyzed by flow cytometry as previously described. Results are expressed as percentage decrease in death=1-((poly-HEMA - control)/(poly-HEMA with mAb - control with mAb)). The top panel represents data from STAD cells (**A**), STAD.APO (**B**) and STAD.ARR (**C**)

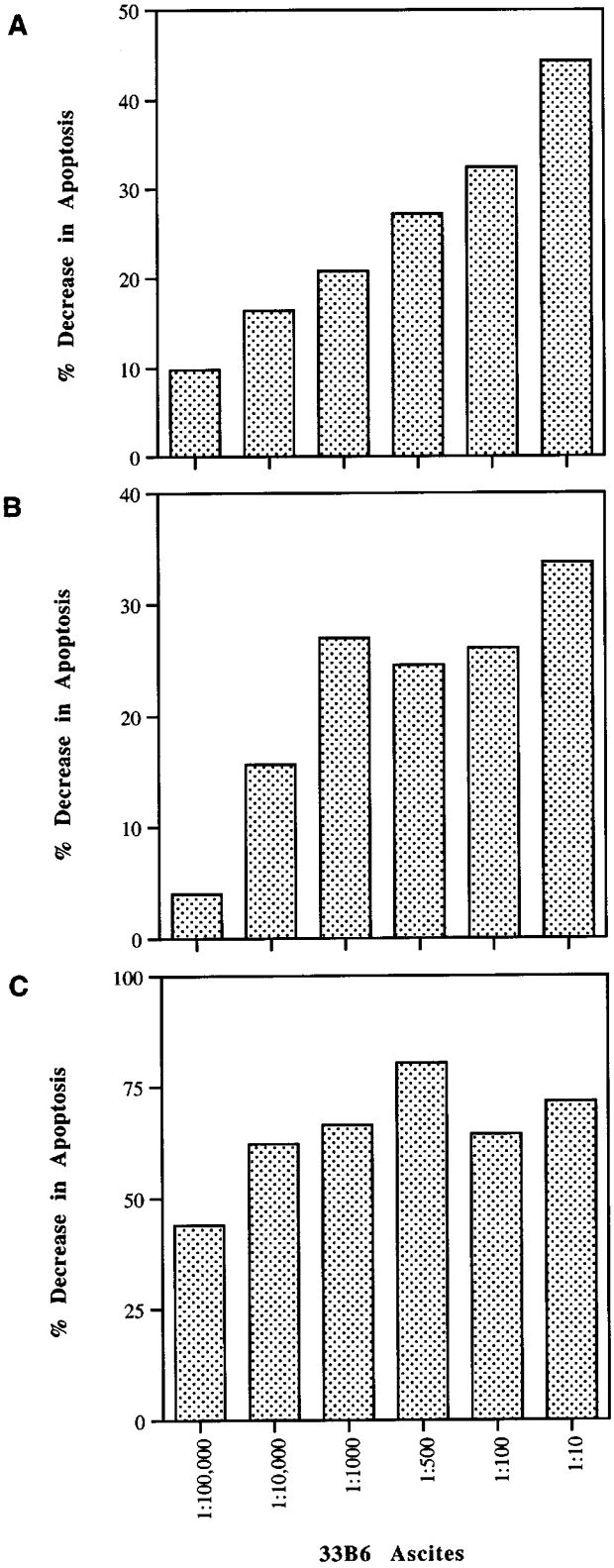


Figure 4 The β1 integrin monoclonal antibody, 33B6 rescues cells from anoikis. 1 : 10 to 1 : 100 000 dilutions of 33B6 ascites were added to 3×10^5 cells per ml at time of plating. Cells were cultured for 24 h in complete media either on untreated tissue culture plastic or on poly-HEMA coated tissue culture plastic. Cells were harvested by standard methods and fixed with ice cold 70% ethanol overnight at 4°C. Cells were stained with propidium iodide and analyzed by flow cytometry. Results are expressed as percentage decrease in death=1-((poly-HEMA - control)/(poly-HEMA with mAb - control with mAb)). The top panel represents data from STAD cells (**A**), STAD.APO (**B**) and STAD.ARR (**C**)

in Table 2. The addition of soluble 33B6 did not inhibit the adhesion dependent arrest of STAD.ARR cells. The cell cycle distribution of STAD.ARR cells on poly-HEMA with the addition of 1 : 100 33B6 ascites shows 71.1% G1, 14.3% G2 and 14.5% S compared to no 33B6 on poly-HEMA 65.9% G1, 16.5% G2, and 17.6% S. Additionally cell cycle progression was not altered by the addition of 18D3 in STAD.ARR cells on poly-HEMA which showed 75.0% G1, 16.8% G2 and 8.2% S compared to no 18D3 on poly-HEMA which showed 71.5% G1, 15.8% G2 and 12.7% S. Thus, cell cycle progression was not effected by ligation of this epitope on the integrin β1 in addition to the epitope targeted by 33B6, suggesting that for cell cycle progression, at least with these two reagents, antibodies are not sufficient to drive the cell cycle under suspended conditions. Furthermore, as expected, monovalent binding using 33B6 Fab fragments was not successful in preventing cell cycle arrest in STAD.ARR cells. The cell cycle analysis showed no substantial effect of treatment 33B6 Fabs in suspension, 54.7% G1, 12.6% G2 and 32.7% S compared to no Fab treatment in suspension, 62.4% G1, 4.2% G2 and 33.4% S. Thus, these experiments indicate that soluble collagen is able to counter suspension induced cell cycle arrest, possibly because of collagen's ability to aggregate in culture and thus provide a binding matrix that allows for receptor crosslinking. While bivalent and monovalent antibody binding as shown by the 33B6, 18D3 and Fab fragment experiments, is sufficient to rescue from apoptosis, it is not sufficient to effect cell cycle progression in the STAD.ARR cell line. As a whole these data suggest different consequences for integrin engagement in the apoptotic cell lines, STAD and STAD.APO and in the cell cycle arresting line STAD.ARR.

Localization of β1 integrin, actin and FAK

To further investigate the nature of soluble collagen and 33B6 mAb treatments on both STAD.APO and STAD.ARR cells we performed immunofluorescent microscopy experiments to visualize associations of β1 integrin with actin and β1 integrin with FAK under suspended culturing conditions. Figure 8 shows the results of these staining experiments. Figure 8A shows STAD.APO cells stained with an anti-β1 mAb (18D3). mAb 18D3 and mAb 33B6 bind distinct epitopes,[14] therefore mAb 18D3 does not have a competing binding site with 33B6 and was used to insure accurate visualizations of β1 integrin location without any interference that might be caused by treatment with 33B6 or collagen. β1 integrins are shown in red and actin stained with phalloidin-FITC is shown in green. Both reagents show even distribution of β1 integrin and actin as further demonstrated in the overlay (yellow). Treatments with 33B6 and soluble collagen show a similar pattern of staining as the no treatment (NT) condition does, suggesting that

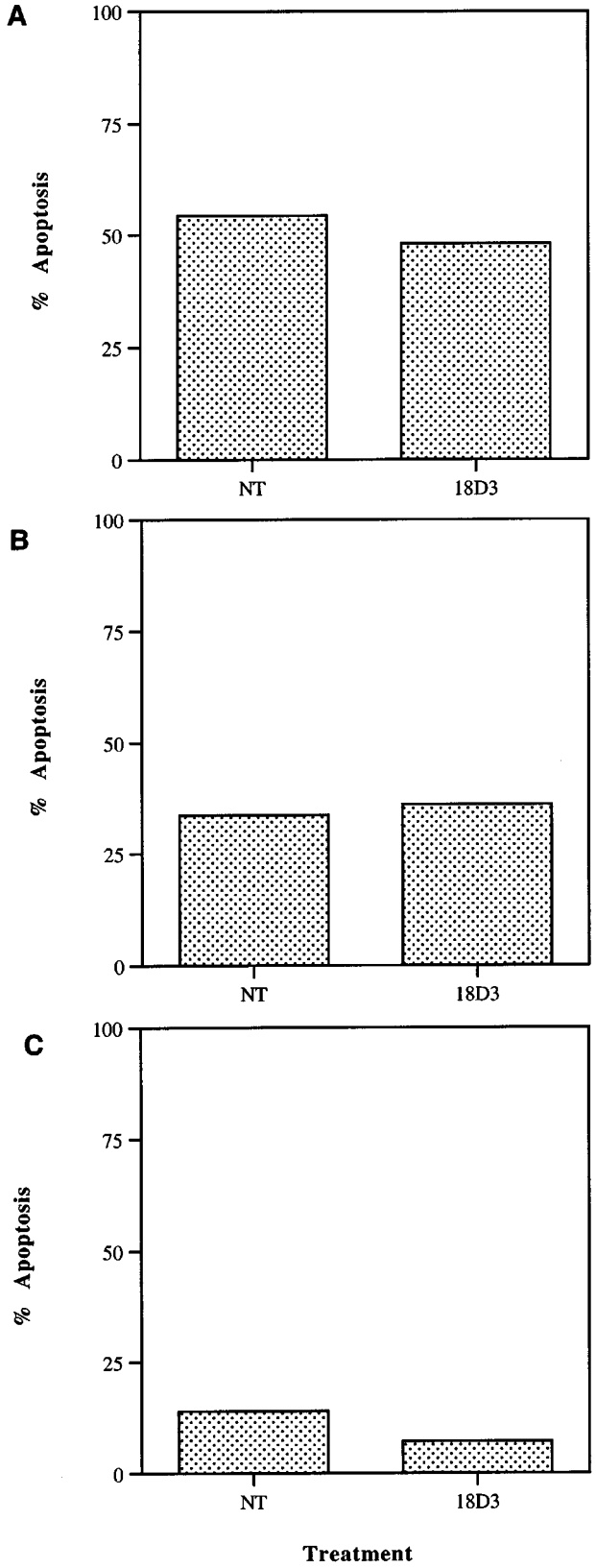


Figure 5 A different anti-ß1 integrin mAb does not save from apoptosis. 1 : 100 dilution of 18D3 ascites was added to 3×10^5 cells per ml at time of seeding. Cells were cultured for 24 h in complete media either on untreated tissue culture plastic or on poly-HEMA coated tissue culture plastic. Cells were harvested by standard methods and fixed with ice cold 70% ethanol overnight at 4°C. Cells were stained with propidium iodide and analyzed by flow cytometry. Results are expressed as percentage decrease in death=1-((poly-HEMA - control)/(poly-HEMA with mAb - control with mAb)). (**A**) Shows results for STAD, (**B**) shows results for STAD.APO, and (**C**) shows results for STAD.ARR in percentage of apoptotic cells

although both 33B6 and soluble collagen are able to save STAD.APO cells from anoikis, neither agent causes redistribution of β1 and actin together into focal adhesion plaques that would indicate integrin clustering. In contrast the same stainings done with STAD.ARR cells in Figure 8B shows that soluble collagen but not 33B6 treatment is able to induce redistribution of β1 and actin into large clusters at the cell surface. This colocalization suggests that integrins cluster in response to collagen treatment but not to 33B6 mAb treatment compared to the no treatment control in STAD.ARR cells.

Further, Figure 8C shows the results of anti-β1 staining with anti-FAK staining to further characterize STAD.APO cells response to treatment to 33B6 and collagen under suspended conditions. Here, we see that 33B6 and soluble collagen do not cause a major redistribution of β1 integrin (red) and FAK (green) into focal adhesion sites. The overlay further shows that β1 and FAK are broadly distributed and not reorganized into focal adhesion plaques that would indicate integrin clustering. However, when STAD.ARR cells (Figure 8D) are stained for β1 and FAK, soluble collagen is shown to be able to induce marked redistribution of β1 integrin and FAK into discrete clusters while 33B6 does not. These results indicate that redistribution of β1 into clusters may be necessary for STAD.ARR cells to re-enter the cell cycle when cultured under suspended conditions while STAD.APO do not have this requirement to be rescued from anoikis.

Discussion

The work presented here focuses on the relationship between integrin ligation and the subsequent effects on cellular behavior. We have created an unique cellular model in which to examine anchorage dependent survival[15] and compare it to suspension induced cell cycle arrest, within the same tumor background. In our model system, the mere absence of adhesion, in the presence of serum and without other exogenous chemical treatments, produces two distinctly different phenotypes in two sublines from a single parental cell line, giving us the opportunity to investigate the effect of suspended culturing conditions resulting in either apoptosis or cell cycle arrest.

To obtain the subclones our strategy was straight forward. Without any manipulation, or selection pressures we performed serial dilutions until a single cell per well was achieved of the STAD cells. These single cells were allowed to expand under standard conditions with complete media until substantial numbers were available to assay for their response to suspended culturing conditions for 24 h. This method was successful in producing STAD.APO, the apoptotic clone and STAD.ARR the cell

cycle arresting clone. To begin characterizing the subclones we performed karyotype analysis. Karyotype analysis revealed through G banding, that there were no discernible differences between STAD, STAD.APO and STAD.ARR lines, and therefore we could not distinguish the varying responses to suspended culturing conditions on a chromosomal level of analysis.

Integrins are primarily responsible for cell adhesion to extracellular matrix therefore to investigate the possible integrin subunit candidates responsible for the differences in response to lack of adhesion, we investigated the integrin cell surface profiles of each cell line. A plausible explanation could have been that because each cell line differed in integrin expression, differential signaling occurred. It has been long established that some malignant phenotypes and their metastatic potential[7] can be attributed to either an upregulation or the loss of certain integrin subunits. The restoration of these malignant cells to their non-malignant integrin expression pattern can in fact abrogate the malignant phenotype. By employing a panel of anti-integrin specific mAbs we stained each clone for two β subunits and seven α subunits to identify which integrins may be responsible for mediating adhesion and if there were any differences in expression levels between clones. The results of these studies revealed that there were no significant differences in the integrin expression patterns between cell lines.

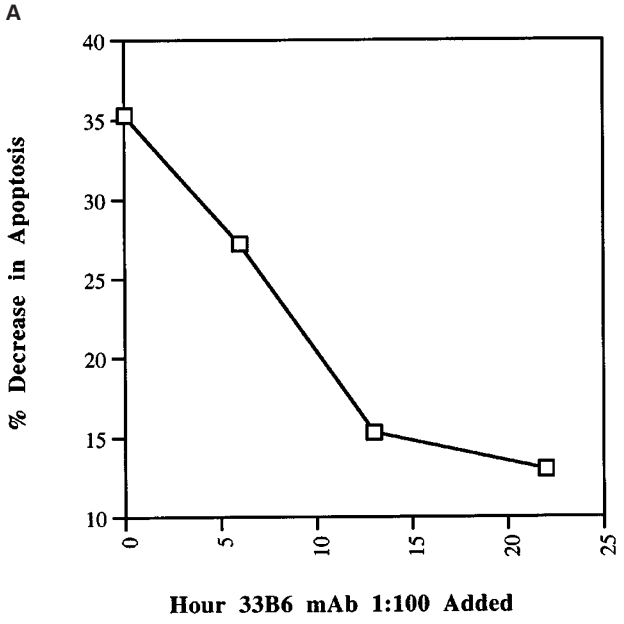
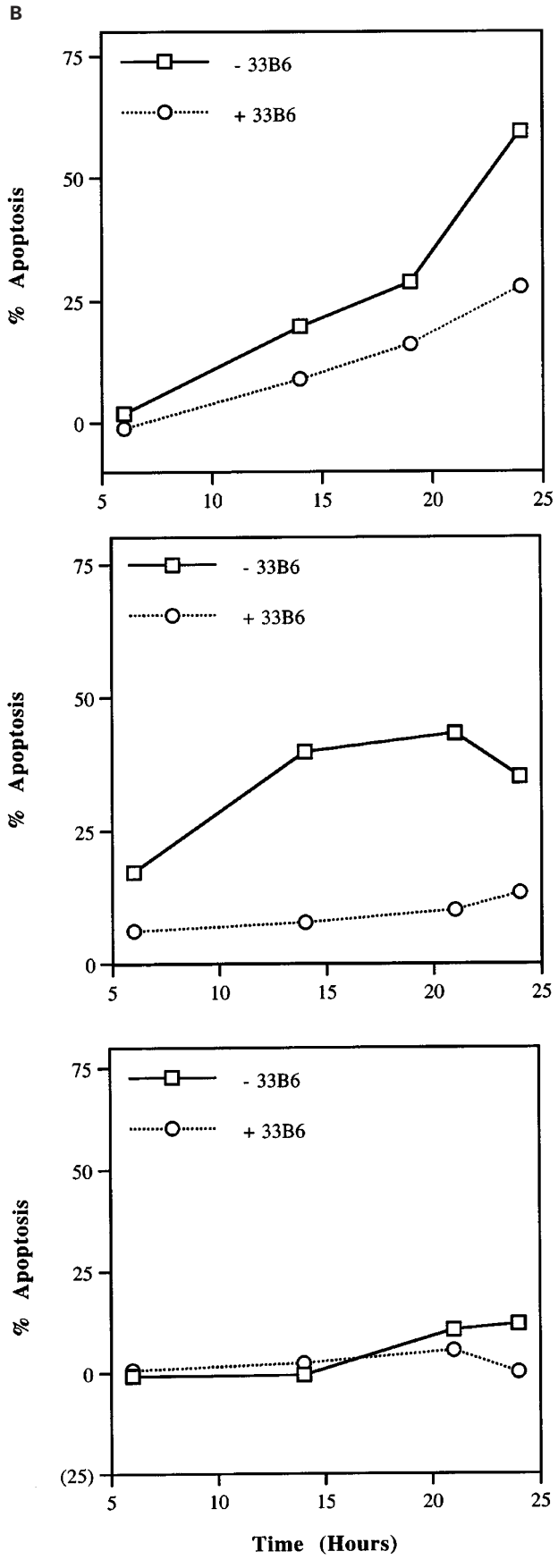


Figure 6 Kinetics of 33B6 effect. 3×10^5 cells per ml were cultured for 24 h in complete media either on untreated tissue culture plastic or on poly-HEMA coated tissue culture plastic. (**A**) Cells were seeded at time 0 and 33B6 ascites at 1 : 100 was added at the times indicated. STAD.APO cells were harvested at 24 h, by standard methods and fixed with ice cold 70% ethanol overnight at 4°C. Cells were stained with propidium iodide and analyzed by flow cytometry. Results are expressed as percentage decrease in death=1-((poly-HEMA - control)/(poly-HEMA with mAb - control with mAb)). In (**B**) cells were seeded in the presence of 33B6 at time 0, and harvested and stained for flow cytometry at the times indicated. Results are expressed in percentage apoptosis. (**A**) STAD, (**B**) STAD.APO and (**C**) STAD.ARR



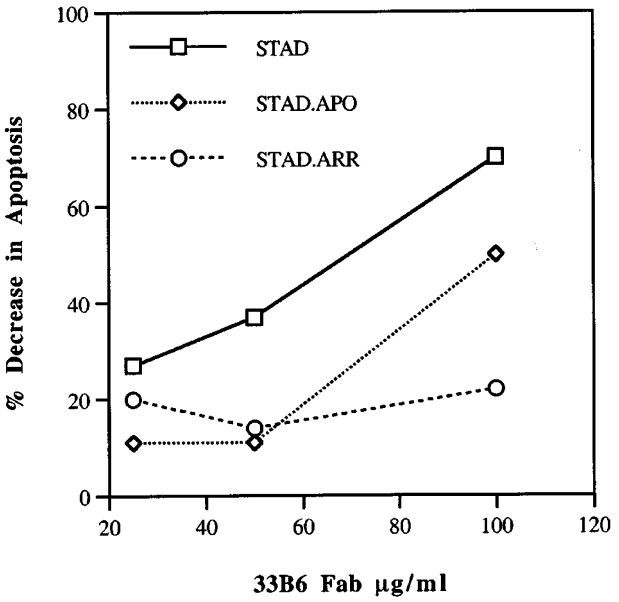


Figure 7 33B6 Fab fragments rescues from apoptosis. A titration of 33B6 Fab fragments was used at 25, 50 and 100 μg/ml, in our standard culturing assay as described previously. Cells were stained with propidium iodide and analyzed by flow cytometry. Results are expressed as percentage decrease in death=1-((poly-HEMA - control)/(poly-HEMA with mAb - control with mAb)). STAD and STAD.APO experiments were performed at the same time while the STAD.ARR experiment was performed separately

Table 2 Cell cycle analysis of STAD.ARR cells in suspension

	% G1	% G2	% S
Soluble collagen			
0	74.5	15.3	10.1
0.1 μg/ml	71.7	15.5	12.8
1.0 μg/ml	59.8	15.2	25.0
10 μg/ml	33.9	25.7	40.4
33B6 ascites			
0	65.9	16.5	17.6
1:100	71.1	14.3	14.5
18D3 ascites			
0	71.5	15.8	12.7
1:100	75.0	16.8	8.2
33B6 Fab			
0	62.4	4.2	33.4
50 μg/ml	54.7	12.6	32.7

3×10^5 STAD.ARR cells per ml were cultured for 24 h in compete media on poly-HEMA coated wells with or without the treatments indicated. Cells harvested and fixed overnight in 70% ethanol at 4°C. Cells were stained with PI, and analyzed by flow cytometry for cell cycle distribution as described in Materials and Methods

Although there were no discernible differences in integrin expression levels between subclones this does not preclude the possibility that they may be utilizing different or several integrins when adhering to ECM. Recently there have been several published results demonstrating that information from one integrin can control the information gained from another integrin on the same cell in a process termed as transdominance or crosstalk.[16–18] Also there existed the possibility that although cell surface expression was present, that in fact these integrin subunits may not be functional due to some intracellular defect either in signaling molecules downstream or a structural defect in the integrin subunit itself. To address these issues we performed a series of adhesion and spreading experiments utilizing immobilized extracellular matrix components and specific anti-integrin mAbs. The adhesion experiments showed that all three cell lines adhered to collagen most rapidly and extensively compared to laminin and fibronectin. Furthermore, the blocking studies demonstrated that adhesion to collagen was most likely mediated through integrin $\alpha 2\beta 1$ and was a phenotypically stable trait of all the clones.

Collagen was demonstrated to be the preferred adhesion substrate for each of the cell clones when immobilized to tissue culture plastic and therefore we asked whether or not soluble collagen would be able to provide a rescuing effect for the apoptotic cell lines, and/or provide the cell cycle arresting line a mechanism by which to re-enter the cell cycle despite suspended conditions. Others have shown[19] using retinoic acid treated neuroblastoma cells that soluble collagen could save cells from apoptosis, under suspended conditions. Our results, albeit it in a drastically different system, confirmed their findings that soluble collagen was able to save from anoikis. In addition, soluble collagen was able to prevent STAD.ARR cells from entering cell cycle arrest. Soluble collagen however can aggregate cells (data not shown) with the ability to cluster receptors and allows for crosslinking,[20] which does elicit some degree of cytoskeletal rearrangement that could be responsible for the downstream signaling events that result in the prevention of apoptosis[3,21] and cell cycle arrest.

Adhesion dependence for survival via integrin $\beta 1$ led us to investigate what effect the addition of an anti- $\beta 1$ mAb (33B6) to the culturing media might have on both the apoptotic clone and the cell cycle arresting clone. In the case of STAD.APO and STAD cell lines, the addition of mAb 33B6, in contrast to soluble collagen, did not cause cell aggregation (data not shown) and resulted in the rescue from apoptosis, most optimally if added at the time of seeding cells on poly-HEMA. This result is somewhat surprising as we have merely ligated the ß1 integrin without allowing for cell shape change to occur under these culturing conditions. In the previously described studies with neuroblastoma cells,[19] soluble collagen could save from apoptosis but soluble anti-integrin mAbs alone could not. This is consistent with the work of others,[22–25] most notably Ingber *et al.*,[26,27] which suggest that indeed the mere ligation of $\beta 1$ integrin without the subsequent cytoskeleton rearrangements should not have resulted in such a dramatic anti-apoptotic effect as we see in our studies. Ingber's work demonstrates that cytoskeletal architecture and the tension and change in that tension are necessary environmental clues mediated by integrins into cells that allow downstream signaling events to occur. They have shown that by inhibiting cells from spreading yet allowing them to adhere, causes cells to enter cell cycle arrest. Only when these cells are allowed to spread to some threshold amount are they released from arrest. Perhaps that is the case with the STAD.ARR cell line that

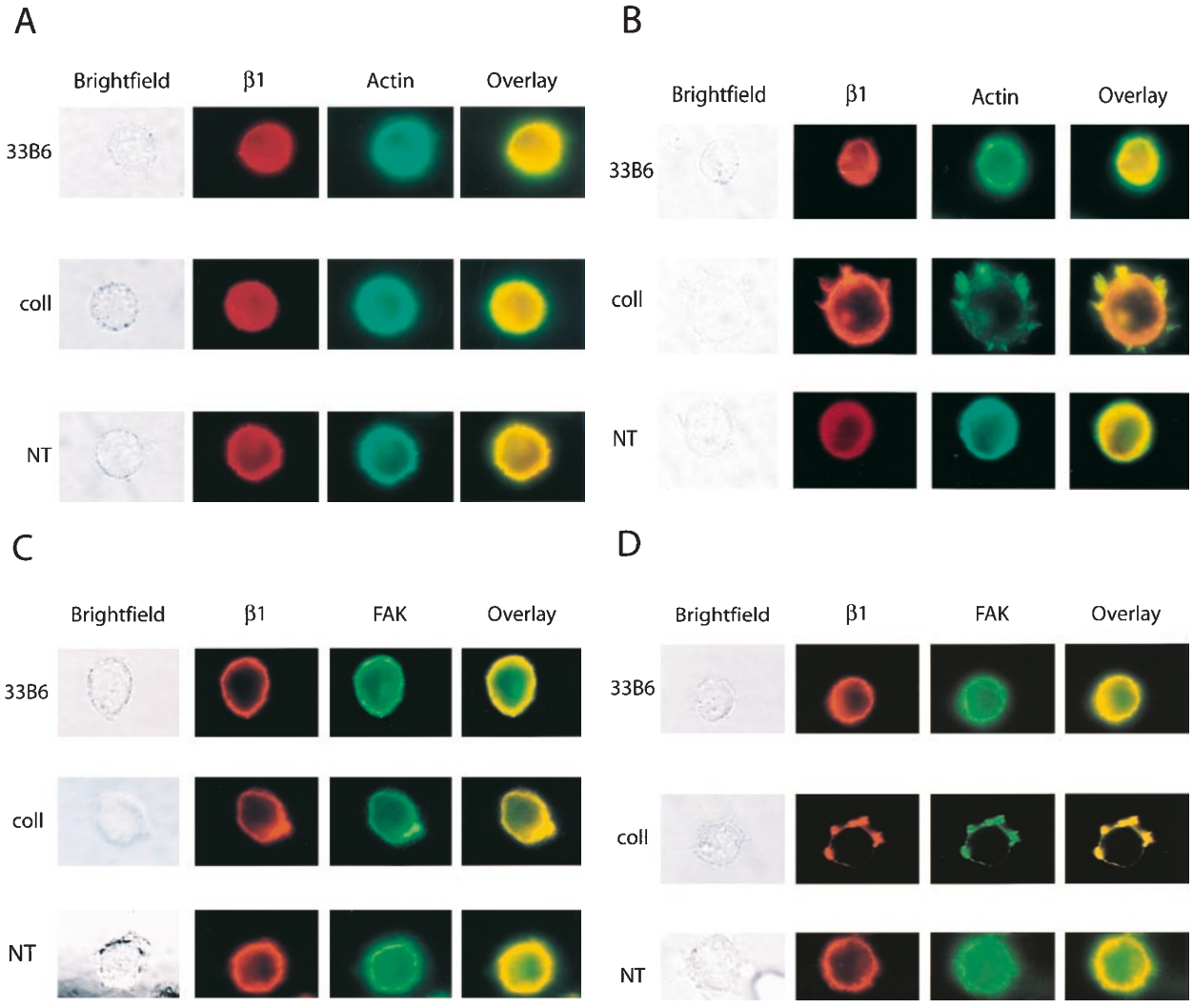


Figure 8 Soluble mAb 33B6 does not cause clustering of β1 integrin with FAK and actin. 1×10^6 cells were incubated for 24 h on poly-HEMA coated wells in complete media in the presence of 33B6 (1 : 100), collagen (10 μg/ml) or without treatment (NT) then harvested, washed, applied to poly-L-lysine coated glass coverslips and fixed with paraformaldehyde. Cells were then stained with the indicated reagents. (**A**) STAD.APO cells and (**B**) STAD.ARR cells were stained with 18D3 anti-β1 mAb (red) and with phalloidin-FITC (green) to stain actin filaments and images were obtained individually for each color. The overlay indicates where the two stainings overlap resulting in a yellow color. (**C**) STAD.APO cells and (D) STAD.ARR cells were stained with 18D3 anti-β1 mAb (red) and with anti- FAK poly-clonal Ab (green) and images were obtained individually for each color. The overlay indicates where the two stainings overlap resulting in a yellow color

showed that the addition of soluble 33B6 did not release the cells from cell cycle arrest as might have been expected given the robust anti-apoptotic effect seen in STAD and STAD.APO.[28] Thus in the work presented here we are demonstrating a scenario where mere ligation of ß1 integrin under suspended conditions can save from anoikis. Furthermore, the situation is more complex in that the ligation of the proper epitope on β1 is required since the addition of the anti-β1 mAb 33B6 saved but the anti-β1 mAb 18D3 had no effect.

Integrin signaling is generally believed to begin after ligation and crosslinking of multiple integrin subunits resulting in clustering of ligated and/or integrin-associated proteins, such that a cell surface signaling complex is formed to set off the downstream signaling cascade.[29,30] To confirm that the bivalent mAb 33B6 was not acting through a crosslinking mechanism, even of only two receptors, we subjected these cell clones to culturing assays with 33B6 Fab fragments. Consistent with our results with intact mAb, 33B6 Fab fragments demonstrated the ability to save from apoptosis in both of the apoptotoic lines and had no effect on the cell cycle arresting line. This result further supports our supposition that in these cells anoikis can be prevented by the ligation of β1 integrin without the extracellular-mediated crosslinking dependent cytoskeletal rearrangements. Yet, the cell cycle arresting line STAD.ARR did not respond to the integrin ligating scenarios with mAbs, suggesting that while adhesion is an important event for both types of cell clones, the cell cycle arresting phenotype requires adhesion and spreading to enter into the cell cycle, and mere β1 ligation will not substitute for true adhesion.[31]

To further investigate the nature of the integrin response to soluble collagen and 33B6 treatments under suspended culturing conditions, we examined whether or not these treatments could cause redistribution of β1 integrin with actin and with FAK into discrete clusters. As shown in Figure 8 STAD.APO cells do not respond to 33B6 nor to collagen by redistributing integrin with actin or with FAK into clusters at the cell surface, in fact we see no effect of these treatments when compared to the no treatment control. Yet, both collagen and 33B6 are able to save STAD.APO cells from anoikis, therefore we conclude that STAD.APO cells do not have a requirement for integrin clustering for signaling to occur to rescue from anoikis.

This differs from the results seen in STAD.ARR cells which show dramatic redistribution of β1 integrin with actin and with FAK in response to soluble collagen but not to 33B6 treatments. Given that collagen can push STAD.ARR cells through cell cycle arrest into the cell cycle and 33B6 can not, and clusters are seen only in the collagen treated samples, we conclude that STAD.ARR cells still retain the requirement for integrin clustering to signal to re-enter the cell cycle under suspended culturing conditions.

Therefore, the differing responses to loss of adhesion in human gastric adenocarcinoma cell lines may represent the differing stages of loss of growth control over non-transformed cells. The apoptotic cell lines STAD and STAD.APO perhaps demonstrate a less dangerous phenotype as they do die when denied adherence as one would expect, however their ability to be rescued without cell shape changes associated with adhesion, or receptor clustering provides a mechanism by which these malignant cells could survive outside of normal cellular growth constraints.[6,32] The STAD.ARR cell line that enters into cell cycle arrest when denied adhesion represents a more dangerous, potentially more aggressive stage of the disease by surviving the loss of adhesion. Because chemotherapeutic agents target cycling cells, STAD.ARR cells would presumably be refractory to chemotherapy drugs and hence would be able to move to secondary sites and establish metastatic lesions. Yet, because ligation of β1 integrin subunit via intact mAb or monovalent mAb did not push arrested cells to re-enter the cell cycle, it suggests that the growth control restriction for cell spreading is still intact, with respect to proliferation. Hence we have obtained a unique and possibly powerful cellular system by which to study integrin involvement in the cellular survival and proliferation.

Materials and Methods

Human cell lines and culturing conditions

The STAD (stomach tumor adhesion dependent) cell line was derived from a parental cell line, ST2[9] that was previously established in this laboratory from a gastric adenocarcinoma tumor. STAD.ARR (cell cycle arrest) and STAD.APO (apoptotic) are sublines that were cloned from the STAD cell line, using standard serial dilution cloning methods. The cell cycle status of single cell clones were then examined to determine apoptosis or growth arrest in response to suspension culturing conditions. All cell lines are maintained in tissue culture flasks containing complete medium (RPMI supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, and 100 U/ml penicillin-streptomycin). Flasks are incubated in a humidified chamber at 37°C in the presence of 5% CO_2.

All experiments were performed with cells at a density of 3×10^5 cells per ml in complete media either on untreated tissue culture plastic or on tissue culture plastic that was coated with poly-HEMA [poly(2-hydroxyethyl methacrylate]) (Sigma, ST. Louis, MO, USA) at a density of 5 mg/cm^2, unless otherwise noted. Poly-HEMA was prepared by dissolving it in 95% ethanol to a concentration of 50 mg/ml and applied to tissue culture wells (1 ml for each of a 6 well plate or 40 μl for each well of a 96 well plate) and allowed to dry at room temperature (RT) for at least 3 h, under sterile conditions in a laminar flow hood.

Cell cycle analysis

Cell cycle distribution was determined by propidium iodide (PI) staining. Cells were cultured under experimental conditions and harvested by first detaching adherent cells with 0.5% trypsin/0.1% EDTA in PBS and washing the cells with PBS. Cells were fixed by resuspension in 1 ml 70% ice cold ethanol and incubated at 4°C overnight. Fixed cells were washed in PBS once before being stained in 200 μl PBS containing 50 μg/ml PI (Sigma, St. Louis, MO, USA) and 25 μg/ml RNAse. Samples were incubated for at least 30 min at 37°C before being analyzed by flow cytometry. Subsequent histograms were analyzed using Multicycle (Phoenix Flow Systems, San Diego, CA, USA).

Cell surface integrin expression

Integrin subunit expression for each cell line was determined by flow cytometry. 3×10^5 cells were suspended in PBS containing 1% goat serum to a volume of 100 μl. Antibodies (either from ascites or purified) were added to each sample. Ascites were used at a final concentration of 1 : 100 and purified mAbs at 10 μg/ml. Samples were incubated for 1 h at 4°C and then washed with PBS/1% goat serum. Pellets were resuspended in 100 μl PBS/1% goat serum. Secondary antibody at a 1 : 100 final concentration of goat anti-mouse IgG conjugated to FITC (Cappell/ICN, Aurora, OH, USA) was added to each sample and they were incubated for 1 h at 4°C. Samples were washed twice with PBS and resuspended in 200 μl of PBS. Samples were then analyzed by flow cytometry on an Epics Profile (Coulter, Hialeah, FL, USA).

Spreading assay

Purified rat collagen type I (Sigma, St. Louis, MO, USA) and laminin from Englebreth Holm-Swarm sarcoma cells (Sigma) were coated onto tissue culture 96 well plates for 3 h under sterile conditions, to a concentration of 10 μg/cm^2. Human plasma fibronectin prepared in this lab according to published procedures[33] was used at 50 μg/ml in PBS and applied to 96 well tissue culture plates. Then the plates were incubated for 4 h in a 5% CO_2 humidified incubator at 37°C. All wells were blocked with 100 μl of sterile 5% BSA (bovine serum albumin) in PBS. Plates were then incubated for 4 h at room temperature or incubated overnight at 4°C. All wells were washed four times with PBS before cells were added. Ten thousand cells per well, in a volume of 100 μl of serum free RPMI media supplemented with ITS (insulin, transferrin and selenium (Gibco, Gaithersburg, MD, USA)), were added after being briefly mixed with one of several blocking antibodies. Ascites were used at 1 : 100 dilution while purified

antibodies were used at 10 μg/ml. 33B6, anti-ß1 mAb ascites, was established and is maintained in our laboratory.[34] All other integrin specific antibodies used are commercially available: A2-IIE10 anti-α2 (Upstate Biotechnology Inc. Lake Placid, NY, USA), P1B5 anti-α3 and P1D6 anti-α5 (Gibco BRL, Gaithersburg, MD, USA), G0H3 anti-α6 (Pharmingen, San Diego, CA, USA).

Soluble ECM culturing assay

Twelve well tissue culture plate wells were coated with poly-HEMA as previously described above or left untreated. In complete medium, 3×10^5 cells were cultured in the presence of 0.1, 1.0 or 10 μg/ml of soluble collagen, laminin or fibronectin for 24 h under standard conditions. Cells were then harvested and stained with PI and then analyzed for cell cycle distribution by flow cytometry.

33B6 Fab fragment production

Purified 33B6 was digested with immobilized papain (0.5 ml, 50% slurry) according to manufacturers' directions (Pierce, Rockford, IL, USA). Immobilized papain was washed twice in a 4 ml volume in digestion buffer (20 mM PO_4, 10 mM EDTA, 20 mM cystiene, pH 7) and resuspended in 0.5 ml digestion buffer. Purified 33B6 mAb was added to the immobilized papain and incubated overnight at 37°C in a shaking incubator. Products of digestion were run over a protein A column equilibrated with digestion buffer and the fall through was collected for the Fab fragments. Purification was verified by 11.25% SDS–PAGE electropheresis. Functionality of Fab fragments was verified by flow cytometry in comparison to undigested mAb.

Immunofluorescent microscopy

Glass coverslips (Corning, NY, USA) were washed with 70% ethanol and air dried under a sterile hood. Coverslips were then coated with 400 μl of poly-L-lysine at 100 μg/ml in PBS and incubated overnight at 4°C. After three washes with sterile PBS the coverslips were blocked with 5% BSA for 2 h at RT and then washed three times with PBS. Cells that were plated under adhered or suspended conditions for 24 h were then harvested as previously described, washed with PBS and resuspended in 200 μl PBS and applied to prepared coverslips and allowed to adhere for 20 min at RT. Cells were fixed with 400 μl of 4% paraformaldehyde for 30 min at 4°C. After washing three times with PBS cells were treated with 0.1% Triton X-100 (Sigma, St. Louis, MO, USA) in PBS for 5 min at RT. Cells were washed three times with PBS and then primary antibody was added, 18D3 (anti-β1 integrin) at 1 μg/ml and anti-FAK (Santa Cruz, CA, USA) 1 : 200 dilution in PBS and incubated for 1 h at 4°C. After washing twice with PBS, secondary antibody was added, rabbit anti-mouse Alexa 594 used at 1 : 250 dilution and goat anti-rabbit Alexa-488 at 1 : 500 dilution and incubated for 1 h at RT. For actin staining, washed cells were stained with phalloidin-FITC (1 : 1000) for 2 h at 4°C. Then cells were washed twice with PBS and mounted to clean glass slides with 40 μl ProLong Antifade (Molecular Probes, Eugene, OR, USA). After curing overnight at RT slides were analyzed by fluorescent microscopy and digital images obtained using Adobe Photoshop 5.0.

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