Journal of Cell Science 111, 1175-1183 (1998)
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JCS1532

Vitronectin regulates smooth muscle contractility via α_v and β_1 integrin(s)

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Accepted 19 February; published on WWW 20 April 1998

SUMMARY

Previous work from this laboratory has established a method for maintaining physiological contractility of dissociated avian smooth muscle in a defined medium at low density. The present report emphasizes the dramatic potency of serum to alter smooth muscle phenotype and induce a loss of contractility. Vitronectin, a molecule purified from plasma, mimicked these effects of serum via an integrin that is RGD-sensitive. Studies utilizing blocking antibodies against vitronectin demonstrated that the presence of this specific adhesion molecule was necessary for the serum-induced loss of contractility. Based on the actions of function-blocking antibodies and RGD-containing peptides, the integrin $\alpha_v\beta_1$ appears to be the primary receptor involved in vitronectin's ability to induce phenotypic transformation in amniotic smooth muscle. The influence of vitronectin on smooth muscle contractility is particularly relevant, because this molecule is abundant in whole blood and plasma (approx. 400 μg/ml). The results suggest that smooth muscle needs to be continually protected from normal blood constituents in vivo. The implications of these results for smooth muscle-related diseases like atherosclerosis, restenosis and Kaposi's sarcoma are discussed.

Key words: Vitronectin, Integrin, Smooth muscle, Contractility, Serum

INTRODUCTION

When smooth muscle cells are dissociated and grown in culture, they usually undergo a profound and well-characterized transformation from a contractile to a non-contractile phenotype (Thyberg et al., 1990). This phenomenon has been observed for sources of smooth muscle cells as disparate as bladder, blood vessels, trachea and avian amnion. In general, the transformation of cultured smooth muscle cells entails not only a loss of contractility, but also a change in morphology, metabolism, adhesive properties, integrin expression and sensitivity to mitogens (Chamley-Campbell et al., 1981; Fowler et al., 1977; Fujio et al., 1993; Halayko and Stephens, 1994; Skinner et al., 1994; Thyberg et al., 1983; Yamamoto et al., 1995). The non-contractile phenotype of smooth muscle is probably an important aspect of the response of smooth muscle to injury, producing what is essentially a wound healing cell/fibroblast (Fowler et al., 1991; Owens, 1995; Ross, 1993; Schwartz et al., 1990). This well-documented transformation is so dramatic and ubiquitous in cultured smooth muscle that one recent review notes, '*...we may know more about the pathological phenotype of the (smooth muscle cell) than we do about its normal state*' (Owens, 1995). Thus, although the 'pathological phenotype' in vitro may be related to the proliferative/fibroid behavior of smooth muscle cells in important clinical settings like atherosclerosis, restenosis and Kaposi's sarcoma (Gravanis and Roubin, 1989; Owens, 1995; Ross, 1993; Steele et al., 1985; Weich et al., 1991), it is clear that studies utilizing differentiated, contractile smooth muscle are needed to explore the etiology of phenotypic transformations. For example, while serum and several matrix/adhesion molecules are known to affect the rate of phenotypic transformation (i.e. loss of contractility) in cultured vascular smooth muscle, it is less clear whether these same molecules can initiate phenotypic transformation (Carey, 1991; Hedin et al., 1988; Libby and O'Brien, 1983; Tagami et al., 1986; Yamamoto et al., 1993; Yau-Young et al., 1981). This is because in most cultured smooth muscle systems, phenotypic transformation is initiated during dissociation or early in culture (Bowers and Dahm, 1993; Thyberg et al., 1990). Studies utilizing contractile smooth muscle are essential for an understanding of the etiology of smooth muscle-related pathologies and complement those studies that examine the behavior of smooth muscle cells after phenotypic transformation has occurred.

The ability to maintain physiological contractility in dissociated amniotic smooth muscle makes this system particularly appropriate for investigating the factors involved in initiating phenotypic transformation (Bowers and Dahm, 1992, 1993, 1994; Dahm and Bowers, 1996). Using stably contractile smooth muscle, the present report establishes the ability of a blood-borne molecule, vitronectin, to induce loss of contractility in cultured smooth muscle cells at physiologically relevant concentrations. This transformation is mediated via a β_1-containing integrin. The $\alpha_v\beta_1$ integrin is specifically implicated. Parts of this work have been previously published as an abstract (Dahm and Bowers, 1996).

MATERIALS AND METHODS

Culture media

Dissociated smooth muscle cells from avian amnion were cultured in N2 medium originally designed for neurons (Bottenstein, 1983). The basal medium was Dulbecco's modified Eagle's medium (DMEM) mixed 1:1 with Ham's nutrient mixture F-12 (F-12) with the following additives (final concentrations): insulin (bovine pancreas; 5 μg/ml), conalbumin (40 μg/ml), sodium selenite (30 nM), progesterone (20 nM), putrescine (100 μM), glutamine (2.5 mM) and ovalbumin (1 mg/ml). The final medium is referred to as N2.

Cell dissociation and culture

Amnions from stage 28-31 white Leghorn chicken embryos (approx. 6 days of incubation) were used for dissociation. Embryos were staged according to the methods of Hamburger and Hamilton (1951). The details of the dissociation have been published previously (Bowers and Dahm, 1992, 1993). Briefly, the anterior third of the amnion was removed from each of three embryos, minced and incubated for 60-70 minutes in 1.5 mg/ml porcine elastase plus 250 units/ml DNase in N2 medium. After rinsing to remove the enzyme solution, the tissue was resuspended in 0.4 ml of Cell Dissociation Solution (Sigma), gently triturated and passed through a 70 μm nylon mesh into 1 ml of N2 medium. Cells were plated at a density of 1×10^4 cells/cm^2 on various substrates (see below) and incubated at 37°C in humidified 5% CO_2/95% air.

Substrates

Acid-washed circular glass coverslips (12 mm diameter) were coated with 0.25-0.50 mg/ml poly(DL-ornithine hydrobromide) (p-orn; M_r, 3000-15,000) as previously described (Bowers and Dahm, 1992), followed by incubation with 1-10 μg/ml vitronectin diluted in DMEM:F12 basal medium for 3 hours at 37°C in humidified 5% CO_2/95% air. A serum substrate was formed by incubating p-orn-coated glass coverslips in 10% fetal calf or human serum/DMEM:F12 for 1 hour at 37°C in humidified 5% CO_2/95% air. Substrate-coated coverslips were rinsed twice with basal medium and incubated in N2 medium for at least 1 hour prior to the addition of cells.

Assay of contractile responses to neurotransmitters

Responses to different neurotransmitter-related substances were monitored visually with a 40× Hoffman objective. Transmitters were applied via pressure ejection from micropipettes with a tip diameter of 3-6 μm. Visual determination of whether a cell contracted in response to drug application was unambiguous and occurred within 1 second of agonist application (Bowers and Dahm, 1992, 1993).

Antibody purification

Chromatography with Protein A-Sepharose (4B-Fast Flow, Sigma) was used to purify IgGs from JG22 and 8E6 ascites, P1F6 and Chav-1 supernatant, and rabbit serum containing antibodies to human vitronectin. IgGs were concentrated using a Centricon-10 concentrator (Amicon, Beverly, MA) with sterile Hepes-buffered Ringer (20 mM Hepes, 127 mM NaCl, 4 mM KCl, 1.6 mM $CaCl_2$, 0.9 mM $MgCl_2$) as the rinsing solution.

Adhesion assay

Cell attachment was measured based on the methods of Neugebauer et al. (1991). Briefly, non-tissue-culture-treated plastic 96-well plates were UV-sterilized and coated with 25 μg/ml laminin or 5 μg/ml vitronectin in sterile Hepes-buffered Ringer (5 mM Hepes, 127 mM NaCl, 4 mM KCl, 0.9 mM $MgCl_2$, 1.6 mM $CaCl_2$, pH 7.2). After overnight incubation at 4°C, each well was incubated with 1% bovine serum albumin in basal medium for 1-2 hours at 37°C to eliminate non-specific binding. Antibodies and arginine, glycine, aspartate-peptides (RGD-peptides) were diluted directly into N2 medium and allowed to equilibrate in the incubator while the cells were being prepared. Amniotic smooth muscle cells or chicken fibroblasts were plated at 30,000 cells/well and the plates were spun at 48 ***g*** for 2 minutes (Eppendorf Centrifuge 5403). After incubation at 37°C for 40 minutes, non-adherent cells were gently washed from the substrate. Adherent cells were fixed for 2 hours at room temperature in 4% glutaraldehyde/Hepes-Ringer, stained with 0.5% Crystal Violet, solubilized with 1% SDS, and quantified by A_{540} (Bodary et al., 1989) in a microtiter plate reader (Molecular Devices). All determinations were carried out in triplicate.

Analysis of intracellular calcium

Indo-1 is a fluorescent derivative of the calcium chelator BAPTA, the emission maximum of which changes from approx. 475 nm in Ca^{2+}-free medium to approx. 400 nm when the dye is saturated with calcium ions (Gryniciewicz et al., 1985). The SFX-2 microfluorimeter (SFX-2; Solamere Technology Group, Salt Lake City, UT 84151) allows the user to measure fluorescent signals and video images simultaneously from a single cell. The 360 nm excitation was delivered to the microscope using a quartz fiber optic bundle, and the objective lens of the microscope was used both to focus the excitation energy onto the specimen and to collect the emitted fluorescence. The emission wavelengths of interest, 410 and 480 nm, were directed to a pair of calibrated photomultiplier tubes (PMTs). The SFX-2 allows fine electronic adjustment of the signal from each PMT to allow compensation for any mismatch in physical properties of the PMTs and in the emission bandwidths, equalizing the efficiency of each channel. Detection was limited to a single cell using an iris at the input port of the detector module. Simultaneous measurement of the signal at each emission maximum allows the ratio of the two values to be determined. With calibration, the ratio can be used to calculate approximate calcium concentration according to the equation of Gryniciewicz et al. (1985). The use of a ratiometric dye makes the measurement of intracellular calcium ion concentration independent of factors such as dye loading, cell thickness, photobleaching by the UV excitation and dye leakage (Diliberto et al., 1994).

Cells were loaded with the cell-permeable acetoxy-methyl ester of Indo-1 by incubating them in N2 medium containing the dye at a concentration of 2.5 μM. The cells were incubated in this solution under normal conditions for 10 minutes, and then rinsed with fresh medium. Unloaded cells had no detectable autofluorescence under the experimental conditions used. Calibration of the calcium signal was performed by determining the maximal and minimal (max-min) dye signals. Because of intercellular variability in the standard max-min calibration, calcium data are presented simply as the emission ratios.

Statistical analysis

When data were dichotomous (e.g. when cells were rated as either contracting or not contracting in response to a ligand), statistical significance was analyzed as the difference between two proportions. Comparisons were always performed within the same dissociation, and at least 100 different cells were analyzed under each condition compared. Proportions were analyzed using the equation $\sigma=[rq(1/n_1+1/n_2)]^{1/2}$, where σ is the standard deviation of the difference between the two proportions, r_1 and r_2 (the difference between the two proportions is assumed to be zero by the null hypothesis; Hoel, 1971). r is the total proportion of cells responding under the combined conditions, and q=1−r. From the above equation, $z=(r_1-r_2)/\sigma$, where z is the number of standard deviations of the actual data from the hypothesized mean (zero). Statistical significance was derived from a table of values for the distribution function for the standard normal distribution. For many experimental conditions, proportions were generated from several different dissociations. Therefore, in the Results section, error bars are sometimes provided to indicate the standard error of the mean between experiments, with *n* referring to the total number of dissociations. However, as stated above, analysis of statistical significance was performed between

proportions within single dissociations. For non-dichotomous data, the two-tailed Student's *t*-test was used to determine statistical significance.

Materials

P-orn, glutaraldehyde, carbachol, culture media, human serum and N2 additives were purchased from Sigma Chemical Co., St Louis, MO. Fetal calf serum was purchased from Hyclone Laboratories, Inc., Logan, UT. Elastase and DNase were from Worthington Biochemical Corp., Freehold, NJ. Bovine vitronectin was purchased from Calbiochem, La Jolla, CA and Gibco BRL. Human vitronectin was a generous gift from Dr Deane Mosher. Laminin was purchased from Collaborative Biomedical Products, Bedford, MA. The GRGDSP and GRGESP peptides were purchased from Peninsula Laboratories, Belmont, CA. Ascites containing JG22 (a monoclonal antibody against the β_1 integrin subunit; Greve and Gottlieb, 1982) was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). Chav-1-producing hybridoma cells and purified monoclonal antibody LM609 were generously provided by Drs Lou Reichardt and David Cheresh, respectively. The cyclic RGD analogue, EMD66203 (c-RGDfV, where f=D-phe), as well as related control analogues, were generous gifts from Merck KGaA (Darmstadt, Germany) via Drs Simon Goodman and Alfred Jonczyk (Eur. Patent EP0578 083; Jonczyk et al., 1995). A monoclonal antibody against the $\alpha_v\beta_5$ integrin (PIF6) was generously provided by Dean Sheppard. A polyclonal antibody against human vitronectin was generously provided by Dr Deane Mosher, and a monoclonal antibody against human vitronectin (8E6) was a generous gift of Dr Helena Hessle. The SL-29 chicken embryo fibroblast cell line was obtained from American Type Culture Collection, Rockville, Maryland. Costar RIA 96-well plates were from Bio-Rad, Temecula, CA.

RESULTS

Previous work demonstrated that the addition of 10% fetal bovine serum to N2 medium caused a dramatic loss of contractility in dissociated amniotic smooth muscle cells (Bowers and Dahm, 1993). The potency of the relevant serum component was not appreciated until cells were plated onto a substrate that had simply been exposed to 10% serum (see Materials and Methods). Smooth muscle cells cultured on 'serum substrate' in N2 defined medium exhibited loss of contractility (Fig. 1) and dramatic changes in cell shape (Fig. 2A,B), similar to those observed in the chronic presence of 10% serum (Bowers and Dahm, 1993). It should be noted that the contractility remaining after 2 or more days on serum substrate is qualitatively different than the contractility exhibited on p-orn. The contractions on serum are primarily local, sluggish, surface protrusions (some spontaneous 'blebbing' is occurring on a few cells in Fig. 2B). By simply counting such cells as contractile, Fig. 1 underestimates the quantitative effects of serum substrate on contractility. The ability of the serum substrate to initiate loss of contractility was blocked by the integrin antagonist, GRGDSP, and not by equal concentrations of its closely related analogue, GRGESP (Fig. 1).

The data above suggested the existence of a factor that is potent or abundant in serum, binds avidly to glass and acts via an integrin. An obvious candidate is vitronectin, known to be abundant in serum and whole blood (100-400 μg/ml), named for its avid binding to glass, and established as an integrin ligand containing an active RGD sequence (Felding-Habermann and Cheresh, 1993; Preissner, 1991). Vitronectin

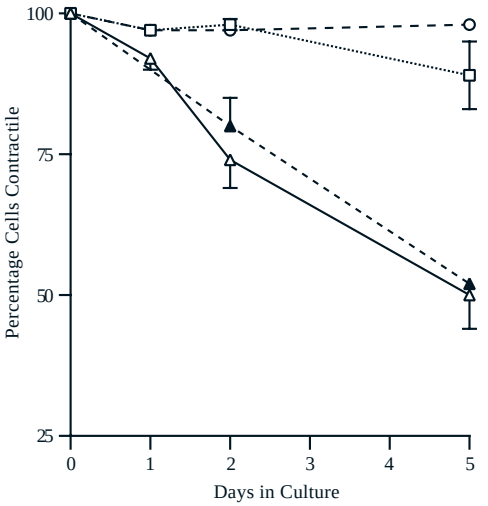

Fig. 1. An integrin antagonist prevents the loss of contractility exhibited by cells grown on a serum substrate. The percentages of dissociated amniotic smooth muscle cells that contracted in response to 100 μM carbachol were assessed after 1, 2, or 5 days in culture on p-orn (○), serum substrate (△, see Materials and Methods for details), serum substrate + 170 μM GRGDSP (□) or serum substrate + 170 μM GRGESP (▲, days 2 and 5 only).

has already been established as the major serum adhesion factor for several cell types (Underwood and Bennett, 1989). Our assay of contractility for smooth muscle cells established that vitronectin causes a loss of contractility similar to that observed with the serum substrate (Fig. 3). Furthermore, cells grown on vitronectin flattened and proliferated in a similar way to those cultured on serum substrate (Fig. 2). The concentrations of vitronectin used in these experiments are well within the range expected for 10% serum. As seen with serum, vitronectin's effect on smooth muscle contractility was fully blocked by the RGD peptide (Fig. 4), indicating the participation of an integrin. Other matrix-associated integrin ligands, like thrombospondin, tenascin and laminin, did not induce loss of contractility (Dahm and Bowers, 1996). Fibronectin caused effects similar to vitronectin on smooth muscle contractility, though the magnitude of the effects was less dramatic (not shown; Dahm and Bowers, 1996).

Contractility is a cytoplasmic event and does not require the movement of a cell's edges to be visually prominent at the single cell level. Thus, the loss of contractility on vitronectin is part of a more global phenotypic transformation and is not simply due to a mechanical restriction of movement. Quantitative documentation of this comes from measures of intracellular calcium after stimulation. Calcium spikes were always absent in cells that did not contract on vitronectin (Fig. 5). After 2 days in culture on vitronectin (10 μg/ml), 38 cells were examined for contractility while simultaneously monitoring intracellular calcium levels. 21 cells contracted and 17 did not, consistent with the results of Fig. 3. The peak calcium levels, as represented by the ratio of emission wavelengths, were 0.85±0.05 (± s.e.m., n=21) for the contractile cells and 0.26±0.01 (± s.e.m., n=17) for the non-contractile cells (P<<0.001; two-tailed Student's *t*-test, corrected for non-identical variances). In contractile cells,

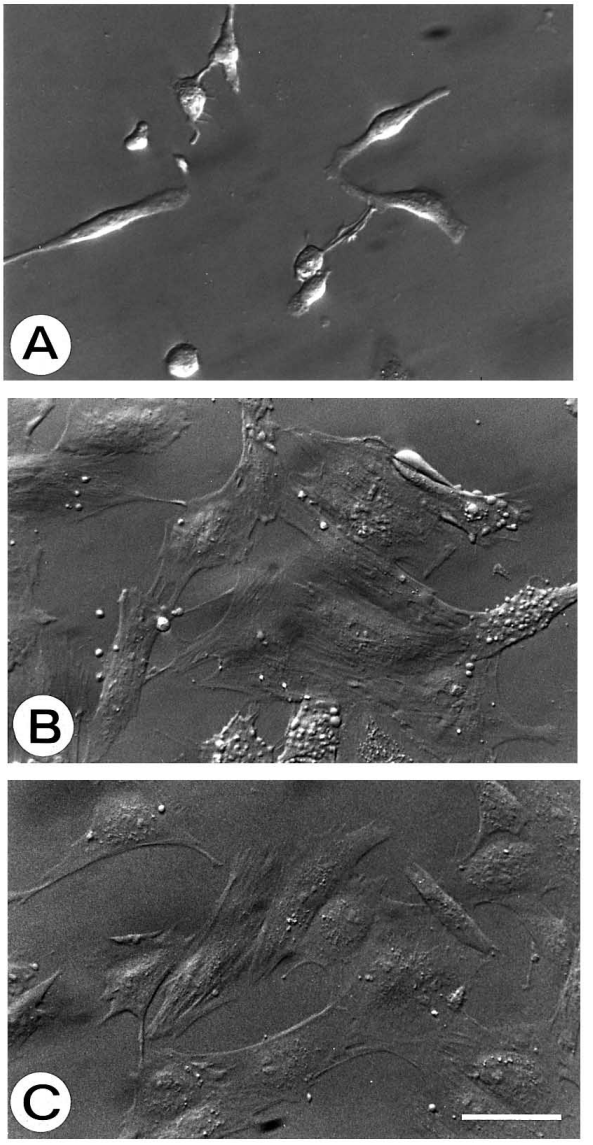

Fig. 2. Smooth muscle cells flatten as they lose contractility. Dissociated amniotic smooth muscle cells were photographed using Hoffman optics after 4 days in culture on (A) p-orn, (B) serum substrate (see Materials and Methods) or (C) vitronectin (5 μg/ml). Bar, 50 μm.

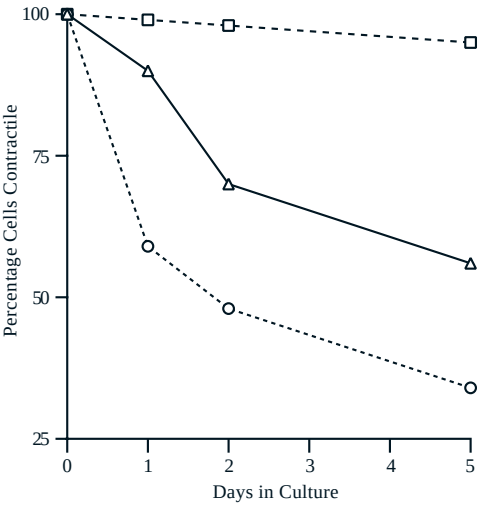

Fig. 3. Vitronectin mimics the serum substrate-induced loss of contractility. The percentages of dissociated amniotic smooth muscle cells that contracted in response to 100 μM carbachol were assessed after 1, 2 or 5 days in culture on polyornithine (□), 1 μg/ml vitronectin (△) or 10 μg/ml vitronectin (○).

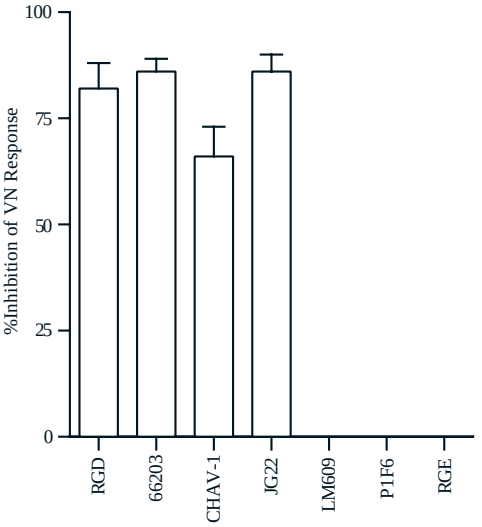

Fig. 4. Loss of contractility on vitronectin is mediated by an $\alpha_v\beta_1$-type integrin. The percentage of cells that contracted in response to 100 μM carbachol after 4 days in culture on 5 μg/ml vitronectin (50±6, mean ± s.e.m., n=4) is taken as the baseline from which the percentage inhibition is calculated. RGD, GRGDSP hexapeptide (170 μM); 66203, cyclic RGD-containing peptide shown to block $\alpha_v\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (5 μM); Chav-1, monoclonal antibody against α_v integrin (200 μg/ml); JG22, monoclonal antibody against β_1 integrin (100 μg/ml); LM609, monoclonal antibody against $\alpha_v\beta_3$ integrin (100 μg/ml); P1F6, monoclonal antibody against $\alpha_v\beta_5$ integrin (200 μg/ml); RGE, GRGESP hexapeptide (170 μM).

intracellular calcium was estimated to increase about three- to fourfold over baseline, whereas little or no increase of intracellular calcium was detectable in cells that did not contract. Peak calcium levels for cells on p-orn were similar to the contractile cells on vitronectin (not shown). The detailed mechanism of this loss of calcium mobilization is not yet fully understood, but it serves to emphasize that the cells on vitronectin are not 'merely' stuck to the substrate. Such a hypothesis would predict normal calcium mobilization with loss of contraction due purely to mechanical resistance. It should also be noted that many cells on p-orn have a flattened morphology with normal calcium spikes and contractions.

Characterization of the integrin that mediates vitronectin's effect was pursued using specific antibodies and RGD reagents. A monoclonal antibody (JG22) that specifically blocks the β_1 integrin subunit (Greve and Gottlieb, 1982) completely blocked the vitronectin-induced loss of contraction in smooth muscle cells (Fig. 4). In addition, an antibody known to inhibit the activity of the α_v integrin subunit in a variety of contexts

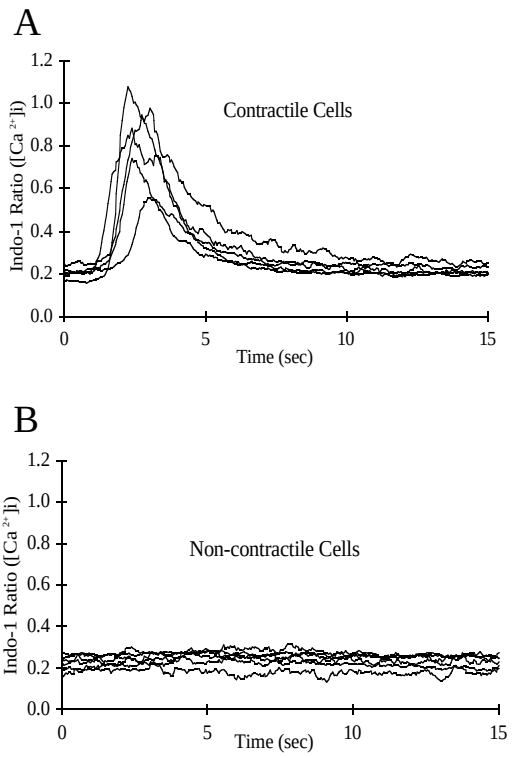

Fig. 5. Loss of contractility on vitronectin is associated with a failure to mobilize intracellular calcium properly. Emission ratios for Indo-1 (indicating intracellular calcium concentration) are shown for (A) five different amniotic smooth muscle cells on vitronectin that contracted and (B) five different cells on vitronectin that did not contract. All cells are from the same dissociation, cultured on the same coverslip, and exposed to a 3-second pulse of carbachol beginning at t=0. For display purposes, every third cell was chosen from the group of 21 contracting and 17 non-contracting cells. Means from analysis of all cells are in the text.

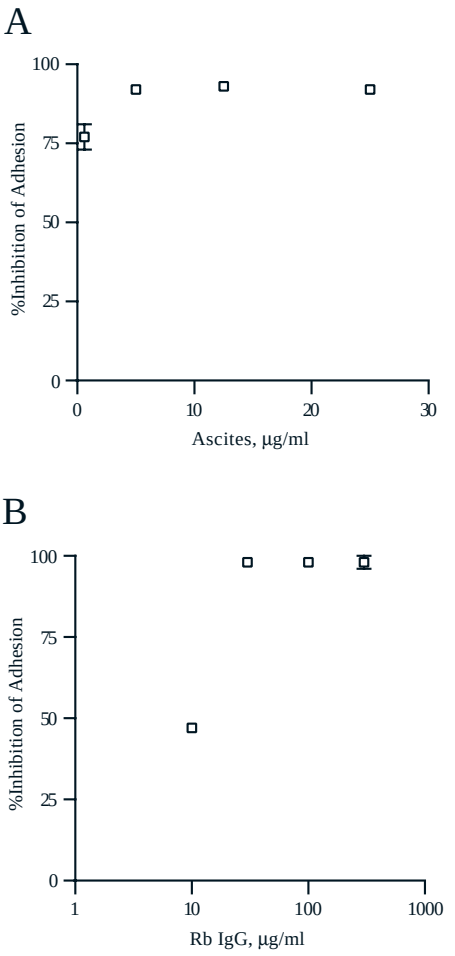

Fig. 6. Antibodies completely block adhesion of chicken SL-29 fibroblasts to human vitronectin. The number of adherent cells on a 5 μg/ml human vitronectin substrate (quantified by A_{540}, see Materials and Methods) was taken as the baseline from which the percentage inhibition of adhesion was determined. Inhibition was maximal at (A) 3 μg/ml IgG in ascites from a mouse injected with 8E6 hybridoma cells and (B) 30 μg/ml purified rabbit (Rb) anti-human vitronectin IgG. Data are presented as the mean ± s.e.m. of 4-5 different wells at each condition; some error bars are too small to be seen.

(Chav-1; Neugebauer et al., 1991) and a recently developed RGD analogue that potently blocks $\alpha_v\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Aumailley et al., 1991; Pfaff et al., 1994; S. Goodman, personal communication) each dramatically inhibited the effects of vitronectin on amniotic smooth muscle (Fig. 4). In contrast, neither a monoclonal antibody against the $\alpha_v\beta_3$ integrin, LM609 (Cheresh, 1987), nor against the $\alpha_v\beta_5$ integrin, P1F6 (Weinacker et al., 1994), blocked the loss of contractility caused by vitronectin (Fig. 4). Both LM609 (Neugebauer et al., 1992) and P1F6 (Friedlander et al., 1995) have been shown to be specific and effective at blocking their respective integrins in avian systems. Thus, agents that block either α_v or β_1 subunits effectively block the action of vitronectin on smooth muscle contractility, while antibodies against the $\alpha_v\beta_3$ or $\alpha_v\beta_5$ complexes do not. These data implicate the $\alpha_v\beta_1$ integrin as the primary mediator of vitronectin-induced loss of contractility in amniotic smooth muscle. In other systems, integrins of the α_v class are known to mediate a variety of effects of vitronectin (Delannet et al., 1994; Hynes, 1992).

Both polyclonal rabbit and monoclonal mouse antibodies (8E6) directed against human vitronectin were obtained to determine whether (1) contaminants in vitronectin preparations might be responsible for the effects observed and (2) whether vitronectin in serum was necessary to induce the loss of contractility caused by serum substrate. Preliminary studies made it clear that human serum had the same effects on chicken smooth muscle as bovine serum. In addition, adhesion studies confirmed that both an antiserum against human vitronectin and the monoclonal antibody (8E6; Hayman et al., 1983) completely blocked adhesion of SL-29 chicken fibroblasts to human vitronectin (Fig. 6). Commercially available chicken fibroblasts were chosen simply as an abundant, reliable source of avian cells to establish the effective dose ranges for the anti-vitronectin antibodies. At comparable concentrations, these same antibodies completely blocked the ability of vitronectin to induce loss of contractility in the smooth muscle cells (Fig. 7). Although the two types of antibodies appear similar when assayed at 2 days, the polyclonal antiserum led to complete

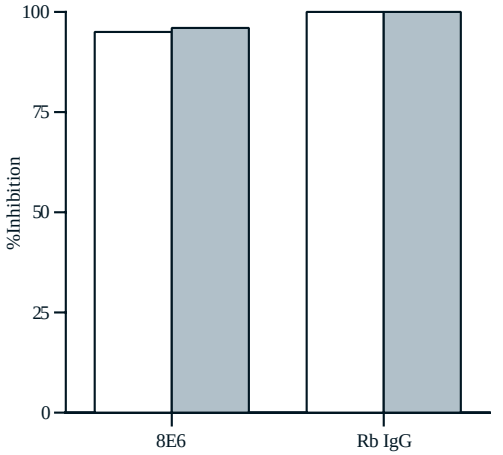

Fig. 7. Loss of contractility on serum-substrate is mediated by vitronectin. The percentages of cells that contracted in response to 100 μM carbachol after 2 days in culture on 5 μg/ml human vitronectin (open bars) or 10% human serum substrate (tinted bars) are taken as the baseline from which the percentage inhibition is calculated. Purified mouse anti-human vitronectin IgG (8E6, 30 μg/ml) or purified rabbit (Rb) anti-human vitronectin IgG (100 μg/ml) completely blocked the loss of smooth muscle contractility.

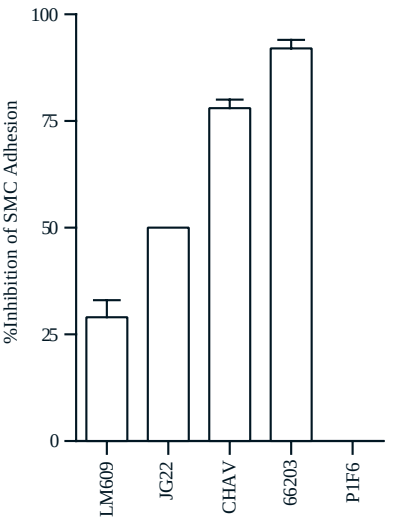

Fig. 8. Smooth muscle cell adhesion to vitronectin is mediated by α_v-containing integrins. The number of adherent cells on 5 μg/ml vitronectin (quantified by A_{540}, see Materials and Methods) was taken as the baseline from which the percentage of adherent cells was calculated. RGD analogue-66203 (5 μM) and Chav-1 (200 μg/ml) each caused a nearly complete block of adhesion to vitronectin, while JG22 (100 μg/ml) and LM609 (100 μg/ml) only partially blocked adhesion to this substrate. Error bars represent the s.e.m. of 3 separate experiments.

detachment and death by 3-4 days in culture, while the monoclonal antibody allowed continued cell adhesion during this time and prevented loss of contraction for those 4 days (not shown). (It should be noted that the adhesion assay of Fig. 6 assesses the ability of cells to attach to a substrate over a period of only 1 hour, which is a very different biological situation from the ability of cells to attach to substrate over a period of many hours or days, as in Fig. 7. The latter situation allows more opportunity for cells to synthesize and alter their immediate environment/substrate.) Importantly, despite the complexity of serum, either antibody could completely block the effect of human serum substrate on smooth muscle contractility, demonstrating that the presence of vitronectin in this system was both necessary and sufficient with respect to the effects of serum (Fig. 7). As with the vitronectin substrate, the monoclonal (but not the polyclonal) antibody allowed the cells to adhere continuously to serum substrate, yet blocked loss of contractility for at least 4 days.

A characterization of the acute adhesion of freshly dissociated amniotic smooth muscle cells to vitronectin allows comparisons between the receptors involved in adhesion versus those involved in the loss of contractility. RGD-66203 and Chav-1 each cause an almost complete block of smooth muscle adhesion to vitronectin, while JG22 and LM609 only partially block adhesion to this substrate (Fig. 8). The effects of these integrin reagents on adhesion are specific for different substrates, as JG22 completely blocks adhesion of amniotic smooth muscle cells to laminin (209±34 versus 15±1 on laminin versus laminin + 100 μg/ml JG22, mean ± s.d., n=3), and 66203 has no effect on adhesion to collagen IV (not shown). The results with vitronectin and adhesion differ from those with loss of contractility, particularly with respect to the efficacy of JG22, the β_1 blocking antibody. Unlike the data on loss of contractility, the adhesion data clearly indicate that more than one α_v-containing integrin is involved in adhesion of amniotic smooth muscle cells to vitronectin. It is noteworthy that these smooth muscle cells possess yet another RGD-sensitive integrin that specifically regulates their responsiveness to a neuropeptide, namely substance P; this integrin is not blocked by either JG22 or the α_v reagents (Dahm and Bowers, 1996). No effect of the P1F6 antibody has been observed in our hands with these cells.

DISCUSSION

While specific aspects of smooth muscle phenotype vary significantly between different tissues (e.g. electrical coupling and neurotransmitter receptor expression), the loss of contractility observed in culture and the associated changes in morphology are documented for every source of smooth muscle examined (Chamley-Campbell and Campbell, 1981; Chamley-Campbell et al., 1979; Halayko and Stephens, 1994; Sas and Miller, 1988; Thyberg et al., 1990). Methods have been established that maintain the contractility of amniotic smooth muscle cells for several weeks in culture, but even these cells exhibit a rapid and total loss of contractility if exposed to mechanical trauma, non-specific proteases or serum (Bowers and Dahm, 1993; present report). These data indicate that amniotic muscle has the same propensity to phenotypic transformation as is observed for other smooth muscle cells, including those of the vasculature. It should be emphasized that loss of contractility in the presence of vitronectin is not simply due to increased attachment to the substrate, preventing, in some simply mechanical sense, the ability of the cells to move. Under 400× Hoffman (and phase) optics, subtle cytoplasmic movements are visible that in no way require a movement of

the 'edges' of the cell. When a cell is categorized as non-contractile, no movement is observed upon exposure to transmitter. In addition, we document in the present study that non-contractile cells on vitronectin have lost their ability to generate intracellular calcium spikes in response to agonist stimulation. These observations demonstrate that loss of contractility of amniotic smooth muscle cells on vitronectin involves much more than simple mechanical restriction of motion.

The results with specific integrin antagonists indicate that the vitronectin-induced loss of contractility is mediated primarily, if not solely, via β_1-containing integrin(s). Because either β_1 or α_v antagonists block the loss of contractility, the most parsimonious conclusion is that $\alpha_v\beta_1$ integrin mediates the vitronectin-induced phenotypic transformation. This latter conclusion is tentative as there is no blocking antibody or other antagonist yet available that is specific for $\alpha_v\beta_1$. That the α_v and β_1 integrin subunits are present on these cells is strongly supported by the blocking data. The α_v and β_1 are among the most promiscuous of the integrin subunits (each capable of interacting with several different integrin subunits), and there is no precedent for their presence in a cell without their association with each other (Bossy and Reichardt, 1990; Clyman et al., 1990; Hynes, 1992). On the other hand, we cannot presently rule out the possibility that loss of contractility results from a combination of integrins each of which is necessary, but not sufficient, to induce phenotypic transformation. For example, the $\alpha_1\beta_1$ and $\alpha_8\beta_1$ integrins are associated with smooth muscle in vivo and would be blocked by the JG22 antibody (Belkin et al., 1990; Bossy et al., 1991; Glukhova et al., 1993; Schnapp et al., 1995a, 1995b). If such a β_1 integrin and an α_v-containing integrin are both necessary for vitronectin's effects, this would account for the present data. One likely candidate in such a scenario, the $\alpha_v\beta_3$ integrin, has been ruled out as a major influence mediating vitronectin's effect on smooth muscle cells in this system using LM609. This conclusion is based on the antibody's inability to block loss of contractility even though it can partially block adhesion in the same cells. The P1F6 antibody has been shown to be active in certain avian systems (Friedlander et al., 1995), but the adhesion data in the present report suggest little, if any, $\alpha_v\beta_5$ in the smooth muscle of amnion. Biochemical characterization of the integrins expressed by amniotic smooth muscle is needed to confidently interpret the P1F6 data.

Serum is a pathological fluid, containing factors not normally present in plasma or extracellular fluid (e.g. platelet factors), whereas vitronectin is present at high (100-400 μg/ml) concentrations in normal blood and plasma (Felding-Habermann and Cheresh, 1993; Preissner, 1991). The presence of a factor in normal blood that can initiate pathological changes in smooth muscle cells suggests that smooth muscle needs to be constantly protected from the factor's influence. Elegant measures of the diffusion barrier across vascular endothelium demonstrate that the endothelium is normally capable of providing significant (though not absolute) isolation of vascular muscle from blood-borne molecules (Matsuki et al., 1993; Rivers and Duling, 1992). Vascular smooth muscle normally expresses abundant β_1 subunit, including the $\alpha_v\beta_1$ integrin, and would be expected to respond to vitronectin if exposed (Belkin et al., 1990; Mechtersheimer et al., 1994; Skinner et al., 1994). The $\alpha_v\beta_3$ integrin, which is also capable of interacting with vitronectin, appears to be inductively expressed under conditions of vascular remodeling, but is low or absent in quiescent vascular muscle in vivo (Brooks et al., 1994; Clyman et al., 1992).

The presence of high concentrations of vitronectin in blood could have significant implications for both normal and pathological aspects of cardiovascular function. Studies in vivo have demonstrated that intimal smooth muscle cells (i.e. those closest to the lumen of the blood vessel) of large arteries have many characteristics of immature or transformed smooth muscle (Glukhova and Koteliansky, 1995). An obvious possibility from the present work is that blood-borne transforming factors like vitronectin leak to some extent across the endothelium into the intimal area, with the muscle closest to the lumen most affected. This situation could account for the stable population of smooth muscle cells in the intima that express decreased differentiated properties (see, for example, Glukhova and Koteliansky, 1995). Locations of these intimal smooth muscle cells correlate with areas of increased endothelial permeability in vivo, consistent with blood-borne factors being critical elements of intimal formation (Gerrity et al., 1977a,b). If the endothelial diffusion barrier is compromised by significant pathology, as occurs for example in AIDS, angioplasty and atherosclerosis (see Xiu et al., 1991; Zhang et al., 1989), vitronectin will have increased access to vascular muscle. Vitronectin is abundant in atherosclerotic plaques, but absent or at very low levels in healthy large arteries (Mori et al., 1995; Sato et al., 1990; van Zanten et al., 1994), and data suggests that the cells of the AIDS-related Kaposi's sarcoma may be derived from smooth muscle (Weich et al., 1991; Wittek et al., 1991). Though speculative, this context may be useful in exploring aspects of the etiology and progression of diseases involving phenotypic transformation of smooth muscle and loss of growth control. Importantly, if clinically relevant pathologies can result from exposure of smooth muscle to vitronectin, the RGD-containing peptides offer a possible course of action with regard to rational intervention in vivo, i.e. treatments utilizing specific antagonists to vitronectin, including those against $\alpha_v\beta_1$. Such integrin antagonists have recently been reported to diminish the proliferative response of vascular smooth muscle after endothelial damage (Choi et al., 1994).

In summary, vitronectin is abundant in both plasma and serum and is capable of transforming contractile smooth muscle to non-contractile, proliferative, fibroblast-like cells. The in vivo behavior of smooth muscle after exposure to blood-borne factors like vitronectin is expected to be influenced by the degree and duration of exposure, as well as by interactions with other local factors (e.g. matrix molecules and platelet factors). Replication of the present results in other types of smooth muscle awaits the availability of more general techniques for maintaining contractility in dissociated cell culture. To date, cultures of vascular smooth muscle that maintain physiological contractility have not been reported. Such studies would provide a framework for testing the relevance of vitronectin and the endothelial diffusion barrier to clinically relevant smooth muscle pathology. Rational intervention to inhibit the pathological effects of endogenous vitronectin may be relatively safe, as this molecule does not appear to be necessary for survival (Zheng et al., 1995).

We gratefully acknowledge Marianne Vecchione for help in establishing and conducting adhesion assays, and we thank Dr Kathryn Cross for expert advice and help on intracellular calcium measures and for critical reading of the manuscript. We also thank Jill Flanagan for excellent secretarial assistance in preparation of the manuscript. The work was supported by a grant to C.W. Bowers from the National Institutes of Health (R29-NS33387).

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