Journal of Cell Science 110, 1477-1487 (1997)
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JCS9615

Functional expression of the alpha 7 integrin receptor in differentiated smooth muscle cells

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SUMMARY

Expression of the α7 integrin is developmentally regulated and is thought to be tissue-specific for both skeletal and cardiac muscles. We now report that α7 is also strongly and ubiquitously expressed by various types of smooth muscle, including vascular, gastrointestinal and genitourinary smooth muscles. In addition, α7 was surface-expressed by a number of smooth muscle cell lines that maintained their differentiated phenotype following adaptation to culture. Studies with the mouse 9E11G smooth muscle cell line showed that the α7 integrin mediated both adhesion and motility of these cells on laminin 1 substrates. α7 expression appears to correlate with the smooth-muscle-differentiated phenotype. The multipotential P19 mouse embryonic stem cell line lacks α7 but uses the α6 integrin to adhere to laminin 1. Following retinoic acid-induced P19 differentiation predominantly to the smooth muscle cell lineage, high expression of α7 was detected along with partial dependence on α7 for binding to laminin. The expression of α7 paralleled the induction of smooth-muscle-specific α-actin, as revealed by dual-labeling flow cytometry. In contrast, α7, which initially was highly expressed on the surface of vascular smooth muscle cell explants, was rapidly downregulated in smooth muscle cell outgrowths as they dedifferentiated into their synthetic phenotype. The results indicate that the expression of α7 integrin in smooth muscle cells is associated with their differentiated phenotype and mediates their interaction with laminins.

Key words: Integrin, Smooth muscle, Laminin

INTRODUCTION

Smooth muscle cells (SMCs) follow a well-defined differentiation program that involves coordinated expression of specific genes involved in contractile functions. Mature SMCs are adherent to their envelope of laminin-rich basement membrane and contract in response to physiological stimuli. Vascular SMCs control the tone of vessel wall by their interaction with the surrounding extracellular matrix. However, the vascular SMC retains its plasticity for rapid vasculogenesis in response to environmental stress (Owens, 1995). SMC surface integrins must play an important role in dynamic adhesive interactions with the components of the extracellular matrix, which range from maintaining their well-differentiated status for contractile function to signalling to induce proliferation, migration and secretion of matrix of vessel walls. Smooth muscle cells express integrin receptors that mediate these interactions (reviewed by Abedi and Zachary, 1995). During atherogenesis, SMC outmigration and neointimal formation also involve integrin-substrate interaction.

In vivo, SMCs have been shown to express a diverse set of integrins that is species- and tissue site-specific. In vascular SMCs, α1, α3, α5, α8, αv, β1 and β3 have been detected (Clyman et al., 1996; Damjanovich et al., 1992; Glukhova et al., 1993; Palmer et al., 1993; Pasqualini et al., 1993; Schnapp et al., 1995; Skinner et al., 1994). Although in large vessels α1 was expressed at high levels in human SMCs, it was not detected in their murine counterparts (Gotwals et al., 1996). Curiously, the α2 and α6 laminin-binding integrins, which are expressed by a number of tissues, are not detected in SMCs found in vivo (Thorsteinsdottir et al., 1995; Wu and Santoro, 1994). An exception is the myometrium, which had strong staining for α6 integrin (Natali et al., 1992; Terpe et al., 1994). A dramatic reduction of α1 integrin in primary cultures of aortic smooth muscle cells has also been demonstrated (Belkin et al., 1990). Interestingly, α1β1 is detected on human, but not murine, medial SMCs in large vessels, and deletion of the α1 integrin gene in mice did not result in an overt phenotype (Gardner et al., 1996).

α7β1 integrin has been identified as a laminin receptor specifically on skeletal and cardiac myoblasts (Collo et al., 1993; Song et al., 1992; Ziober et al., 1993) and melanoma cells (Kramer et al., 1989). However, reverse transcription-polymerase chain reaction (RT-PCR) results demonstrated a wide range of α7 expression, including stomach, liver and spleen (Collo et al., 1993). A more extensive analysis is required to establish the expression pattern of this integrin. In this study, we found by immunocytochemistry that α7 is expressed not only in striated

muscles but also in vascular, gastrointestinal, and genitourinary SMCs. In addition, α7 was detected on the cell surface of early passages of primary vascular or uterine SMCs. Moreover, surface expression of α7 integrin was biochemically detected in established SMC lines, and the integrin mediated both cell adhesion and migration on laminin 1 substrates.

MATERIALS AND METHODS

Cell culture and reagents

The 9E11G mouse SMC line and R21696V9 rat primary SMC line were kindly provided by Dr Gary Owens (University of Virginia, Charlottesville, VA) and maintained in α-MEM with 7.5% fetal bovine serum (FBS) or in 50% F12, 50% Dulbecco's minimum essential medium (DMEM) with 10% FBS, respectively. The detailed characterization of 9E11G as a smooth muscle cell line has been described (Blank et al., 1995). The PAC1 rat pulmonary SMC line (kindly provided by Dr Abraham Rothman, University of California, San Diego, CA) was maintained in DMEM H-21 with 10% FBS (Rothman et al., 1992). P19 cells were obtained from the Cell Culture Facility (University of California, San Francisco) and maintained in α-MEM containing 10% FBS. Laminin 1 was purified from mouse EHS tumor as previously described (Kramer et al., 1991).

Antibodies against integrin subunits included hamster anti-mouse and rat α1 monoclonal antibody (mAb) Ha31/8, anti-mouse and rat α2 mAb Ha1/29 and anti-mouse and rat β1 mAb Ha2/11(Yao et al., 1996b), kindly provided by Dr Donna Mendrick (Human Genome Sciences, Rockville, MD). The Ha1/29 mAb reacts to the mouse α2 integrin subunit in immunoprecipitation and differentiation assays in mouse osteoblasts (C. H. Damsky, personal communication). Rabbit anti-mouse α3 antiserum was kindly provided by Dr Hannu Larjava (University of British Columbia, Vancouver, Canada). Rat anti-mouse α5 mAb IIA1 was purchased from Pharmingen (San Diego, CA); rat anti-mouse α6 mAb GoH3 was purchased from AMAC (Westbrook, ME). The rabbit polyconal antibodies (pAb) 22780 and 1211 were against the α7A and α7B cytoplasmic regions, respectively, and were described previously (Yao et al., 1996a). The rabbit pAb 22778 was generated against peptide sequences to β1 cytoplasmic region GENPIYKSAVTTVVNPKYEGK by methods similar to preparing pAb 22780 and 1211. Rat anti-mouse α7 mAbs CA5, CY4 and CY8 were described previously (Yao et al., 1996a). CY8 was used for function-perturbing assays. Monoclonal Abs anti-α smooth muscle actin clone 1A4 and anti-human vinculin clone hVIN-1 were purchased from Sigma. Fluorescein (FITC)-, rhodamine-, and alkaline phosphatase (AP)-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). R-Phycoerythrin-conjugated goat anti-mouse IgG was obtained from Tago (Burlingame, CA). Secondary biotinylated antibodies (either anti-rat or anti-rabbit IgG) were purchased from Vector Laboratories (Burlingame, CA). Streptavidin-horseradish peroxidase (HRP), and enhanced chemiluminescence (ECL) kit were purchased from Amersham (Arlington Heights, IL).

Immunoprecipitation and western blotting of tissue lysates

A Balb/c mouse was killed by cervical dislocation, and tissues were dissected and frozen in liquid nitrogen. Frozen tissues were then minced and solubilized in lysis buffer (200 mM octyl-β-D-glucopyranoside, 50 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 2 mM phenylmethylsulfonyl fluoride, 1 mM *N*-ethylmaleimide, 2 mM leupeptin) for 2 hours. Lysates were precleared with anti-rat agarose beads overnight, and equal amounts of protein were mixed with goat anti-rat agarose for ≥3 hours with mAb CA5 and CY8 to α7. The beads were washed with the wash buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 0.5% Nonidet P-40, 0.1% BSA) three times and heated at 100°C in SDS sample buffer for 5 minutes. The supernatant was divided into two aliquots: one for nonreducing samples and one for reducing with 2-mercaptoethanol. Samples were separated by 7.5% SDS-PAGE under reducing and nonreducing conditions and then transferred to Immobilon-P membrane (Millipore, Bedford, MA). The membranes were incubated with pAb 22780 to α7A or pAb 1211 to α7B and then with AP-conjugated goat anti-rabbit IgG. Signals were detected with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Promega, Madison, WI).

Immunohistochemistry

Mice were killed by cervical dislocation and the tissues were isolated and immersion-fixed for 2 hours in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C. Tissues were immersed in OCT Compound (Tissue-Tek, Miles Inc., Elkhart, IN), frozen in ethanol bath on dry ice and stored at −80°C. Sections (6-10 μm thickness) were collected on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA), air-dried, and blocked with 10% normal goat serum (NGS) overnight. The sections were incubated with primary antibodies CA5 and CY8, diluted in PBS with 10% NGS at room temperature in a moist chamber for at least 1 hour, washed with PBS, and then incubated with FITC-conjugated goat anti-rat IgG. Nuclei were counter-stained with propidium iodide (1 μg/ml). After washing with PBS, the samples were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and observed under a Nikon epifluorescence microscope.

When the avidin-biotin complex (ABC) method was used, sections were quenched with 0.3% hydrogen peroxide before blocking with 10% NGS. Secondary biotinylated antibodies (either anti-rat or anti-rabbit IgG; Vector Laboratories) were used, and then the sections were exposed to avidin-biotin-complex (ABC Elite kit; Vector Laboratories) and reacted with diaminobenzidine according to the manufacturer's recommendations.

Immunoprecipitation of surface biotin-labeled cells

Confluent cultures of cells were washed twice with PBS and then labeled with NHS-LC-Biotin (Pierce), 1 mg/ml in cold PBS at 4°C for 90 minutes. Cells were washed twice with 50 mM glycine blocking buffer and incubated in this buffer for 10 minutes at 4°C. Next, the cells were lysed in lysis buffer (PBS with 0.1 M Tris-HCl, pH 7.5, 2% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride and 1 mM *N*-ethylmaleimide). After preclearing with Protein A beads, the lysate was mixed by rotation for ≥3 hours with primary antibody and Protein A beads. The beads were washed three times with the wash buffer and heated at 100°C in SDS sample buffer for 5 minutes. The supernatant was divided into two aliquots: one for nonreducing samples and one for reducing with 2-mercaptoethanol. Samples were separated by 7.5% SDS-PAGE under reducing and nonreducing conditions. The biotinylated proteins were detected by streptavidin-HRP and then ECL.

Flow cytometry

Subconfluent cells were detached with 2 mM EDTA. Single-cell suspensions of 10^6/ml were incubated with optimal concentrations of primary antibodies in wash buffer (2% normal goat serum in PBS) for 1 hour on ice, washed three times and incubated with the secondary fluorescein-labeled antibodies for 30 minutes on ice. After washing again three times, the cells were stained with propidium iodide (1 μg/ml) to identify non-viable cells. Flow cytometry was performed on a FACscan flow cytometer (Becton Dickinson). Control samples consisted of cells with or without secondary antibody binding. Any non-viable cells stained with propidium iodide were eliminated from the analysis.

For double staining with anti-α7 and anti-α smooth muscle actin mAbs, retinoic acid-treated P19 cells were first single-stained with mAb CY8 (anti-α7) and FITC-conjugated goat anti-rat IgG. They were then fixed with 2% paraformaldehyde in PBS for 10 minutes, washed, quenched with 20 mM glycine for 10 minutes, and permeabilized with 0.4% Triton X-100 for 5 minutes. Cells were then incubated with mAb

1A4 (anti-α smooth muscle actin), washed three times, incubated with R-phycoerythrin-conjugated goat anti-mouse IgG for 30 minutes, and washed extensively before flow cytometry. Control samples consisted of cells stained with or without secondary antibodies and cells single-stained with mAb CY8 or with mAb 1A4 with secondary antibodies to rule out any cross-reactivity among the secondaries.

Immunocytochemistry

Cells were seeded on laminin 1-coated coverslips for 1 hour, washed with PBS and fixed with 2% paraformaldehyde in PBS. Cells were permeabilized with 0.4% Triton X-100 for 5 minutes and blocked with 10% normal goat serum in PBS for at least 1 hour. Samples were incubated with primary antibodies for 1 hour at room temperature, washed with PBS, and then incubated with FITC- or rhodamine-labeled anti-rat or anti-mouse IgG for 1 hour at room temperature. After washing with PBS, the samples were mounted in Vectashield (Vector) and observed under a Nikon epifluorescence microscope.

Cell adhesion assay

Cell adhesion was assayed as described previously (Yao et al., 1996a). The maximum number of cells bound to the substrates at the specified concentration was used to indicate 100% attachment. Background cell adhesion to 1% BSA-coated wells was subtracted. The effect of specific antibody was tested by preincubating the cells with optimal dilutions of purified antibody on ice for 30 minutes prior to the assay.

Migration assay

Cell migration was assayed in a modified Boyden chamber (Neuroprobe, Bethesda, MD) as described previously (Matsumoto et al., 1994). 9E11G cells were serum-starved overnight before the assay. The lower chamber was filled with 0.1% BSA in DMEM with 20 ng/ml platelet-derived growth factor (PDGF)-BB. Migrating cells were stained with 1% Crystal Violet, and nine randomly chosen fields from triplicate wells were counted at ×400 magnification. The effect of specific antibody was tested by preincubating the cells with the optimal dilutions of purified antibody on ice for 30 minutes prior to the assay. Control groups consisted of cells in the absence of antibodies.

Induction of P19 cell differentiation

Induction experiments were performed as described previously (Blank et al., 1995). P19 cells (10^6) were plated on a 10 cm dish and treated for 2 days with 1 or 2 μM retinoic acid (RA). Next, cells were washed and maintained in α-MEM containing 7.5% FBS for 7 days. Cells were then harvested for flow cytometry analysis. Induction experiments were performed separately with 1 or 2 μM RA, and the SMC percentage in each induction was 30% and 70%, respectively. The result from the experiment using 2 μM RA is shown here. Successful induction of SMCs was confirmed by detection of α smooth muscle actin by FACS analysis.

Primary SMC culture

Balb/c mice were killed by cervical dislocation. The descending aorta or uterus was removed and the connective tissues were carefully teased away under a dissecting microscope. Tissues were cut into segments <0.5 mm in diameter and placed on laminin-coated dishes or coverslips. Outgrowth cells were processed for experiments or trypsinized to expand the population 7 to 10 days later. The SMC phenotype of the explant cells was confirmed by their morphology and positive staining with α smooth muscle actin.

RESULTS

Distribution of α7 in vivo

α7 integrin has been described as a skeletal- and cardiac-muscle-specific integrin (Bao et al., 1993; George-Weinstein et al., 1993; Song et al., 1992; Ziober et al., 1993). However, other studies using RT-PCR or in situ hybridization have suggested α7 is expressed in other non-striated muscle tissue or cell lines (Collo et al., 1993; Hierck et al., 1996; Ziober et al., 1993). Therefore, we performed immunoprecipitation of mouse tissue lysates with mAb CA5 and CY8 followed by blotting with polyclonal antibodies specifically against either the α7A or α7B cytoplasmic isoform as described in Materials and Methods. Interestingly, a detectable level of α7A was found only in skeletal muscle (data not shown) whereas α7B was found not only in skeletal and cardiac muscle but also in stomach and lung (Fig. 1A,B). No signal for α7A or B was detected in extract from either liver or spleen. As described in previous studies (Kramer et al., 1989; Yao et al., 1996a), the α7 integrin migrated at 120 kDa under nonreducing conditions (Fig. 1A) and its light chain fragment at 25-35 kDa under reducing conditions (Fig. 1B).

To further define the cell types expressing the α7 integrin, we analyzed tissue expression for α7 integrin in adult mouse tissues, using immunohistochemistry with mAbs CA5 and CY8. As expected, α7 was detected in skeletal muscles at the myotendinous junction (Fig. 2B,C) (Bao et al., 1993; Martin et al., 1996). However, anti-α7 mAbs generated relatively more intense staining in the smooth muscle walls of arterioles and venules than in sarcolemma in skeletal myofibers. In addition, staining was also detected in the perineurium (Fig. 2A). Of interest, smooth muscles in a number of tissues were positive for α7 expression; these include smooth muscle layers in the gastrointestinal (Fig. 2D and F) and genitourinary tracts (Fig. 2E and G), smooth muscle surrounding bronchioles in the respiratory tree (Fig. 2H) and the vascular wall of the descending aorta (see Fig. 9A). Sections of lung containing bronchioles (Fig. 2H), of the middle third of the esophagus containing both skeletal and smooth muscle components (Fig. 2D), and of intestine, bladder and uterine wall are shown here as examples (Fig. 2E-G).

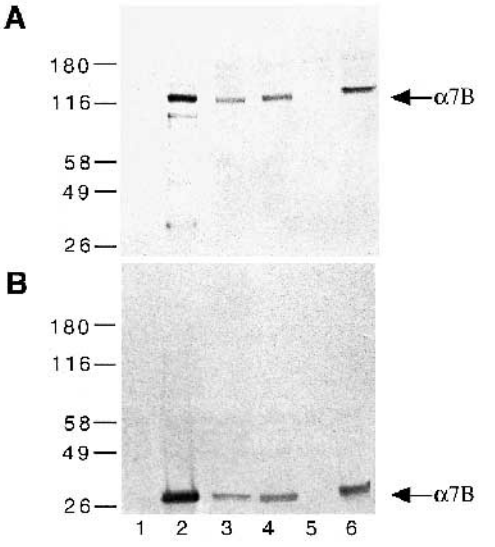

Fig. 1. α7 integrin expression in mouse tissues. Selected mouse tissues were processed for immunoblotting as described in Materials and Methods. Blots were probed with polyclonal antibody 1211 to α7B under nonreducing (A) or reducing (B) conditions. Lane 1, liver; lane 2, heart; lane 3, lung; lane 4, skeletal muscle; lane 5, spleen; lane 6, stomach. The positions of α7 integrin and its heavy and light chains are indicated. Molecular mass standards (in kDa) are indicated at left.

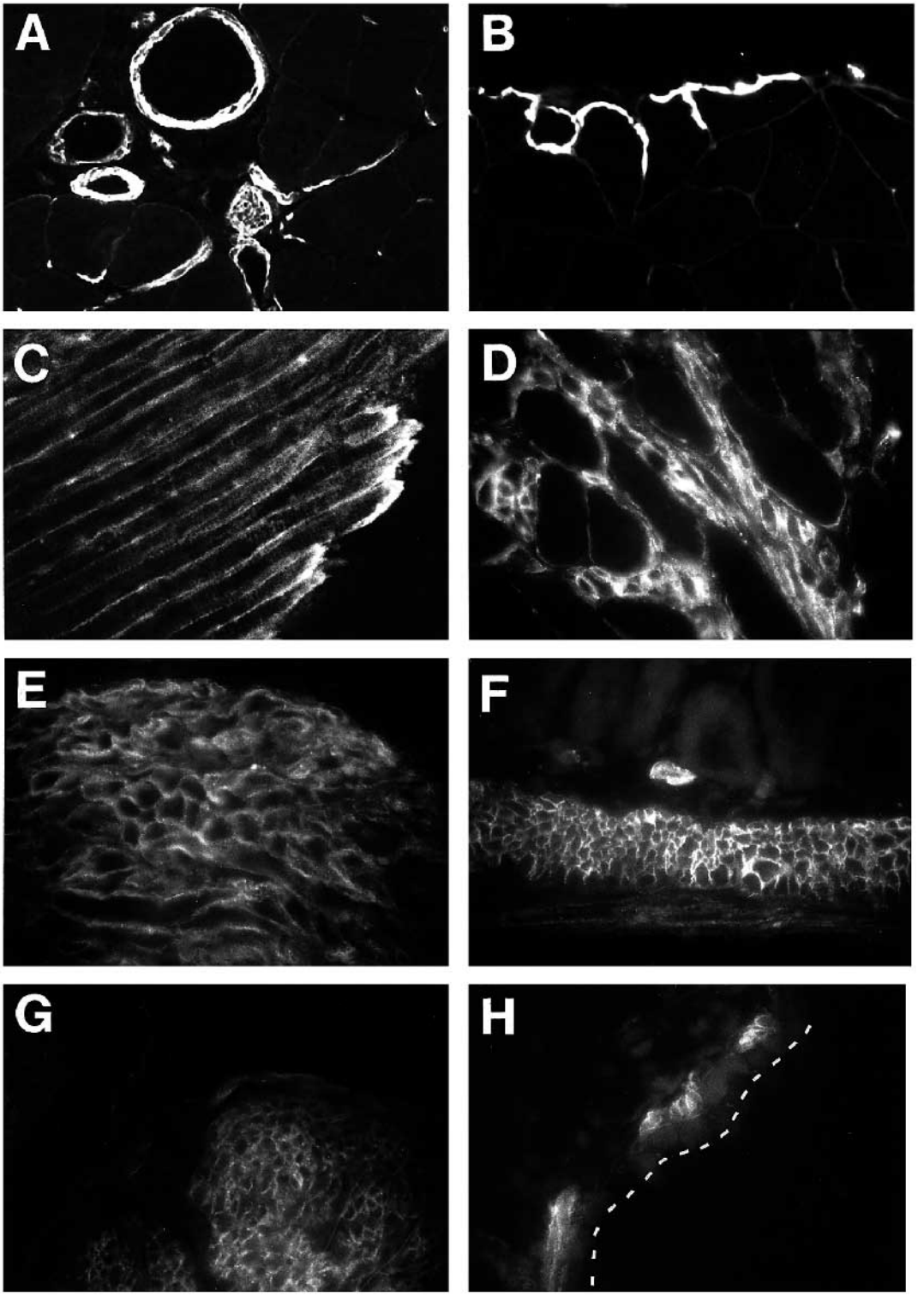

Fig. 2. Immunohistochemical detection of α7 distribution in different tissues. Frozen sections of mouse tissues were stained with mAb CY8 against mouse α7 integrin and secondary goat anti-rat IgG conjugated with FITC as detailed in Materials and Methods. Positive staining of smooth muscle cells was found in (A) blood vessels and perineurium, (B, C) myotendinous junctions in skeletal muscles, smooth muscle layers in (D) esophagus, (E) bladder, (F) intestine, and (G) uterine wall, and (H) bronchioles in lung. Broken line outlines the lumen of the bronchiole.

Expression of α7 integrin by cultured smooth muscle cells

We examined the expression of α7 in a number of well-differentiated murine smooth muscle clonal cell lines: PAC1, R21696V9, and 9E11G (Blank et al., 1988, 1995; Rothman et al., 1992). Immunoprecipitation showed that antiserum 1211 against the 7B isoform detected surface α7 integrin expressed in all three surface-biotinylated cell lysates (Fig. 3A, lanes 1-3). In addition, rat anti-mouse α7 mAb CY8 immunoprecipitated α7 integrin in the mouse 9E11G cell line (Fig. 3A, lane 4). We also tested cultured primary uterine SMCs which were first surface-biotinylated and then immunoprecipitated with mAb CY8. α7 integrin was also detected in these primary uterine cells (Fig. 3A, lane 5). Finally, when PAC1, R21696 or 9E11G SMCs were

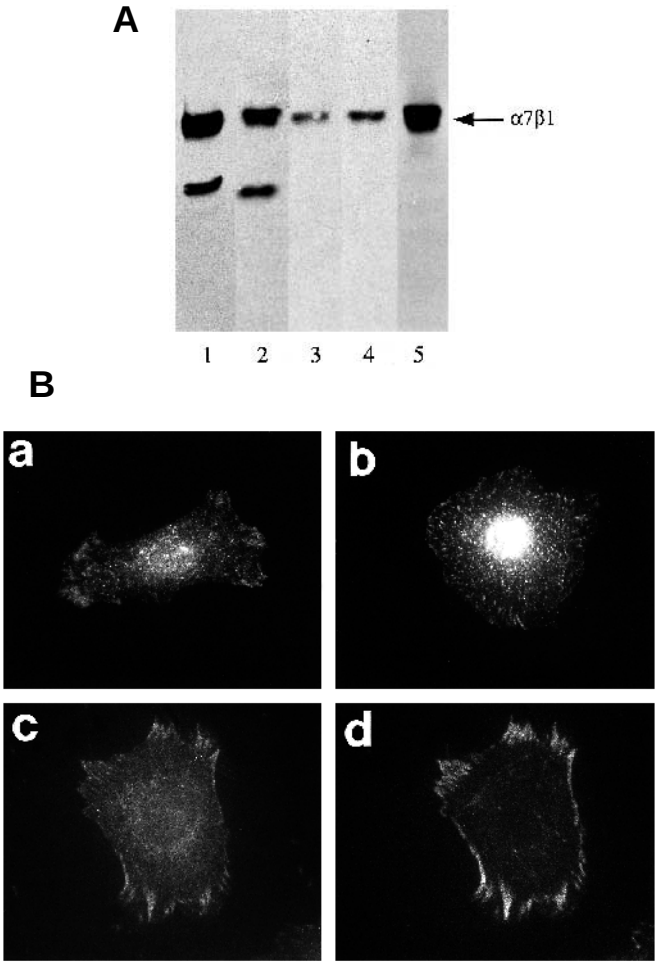

Fig. 3. Expression of α7 integrin in cultured smooth muscle cells. (A) Detection of surface expression of α7 integrin in rat PAC1 (lane 1), rat R21696V9 (lane 2), mouse 9E11G (lanes 3 and 4) and mouse primary uterine SMCs (lane 5). Cells were surface-biotinylated and immunoprecipitated with polyclonal Ab 1211 against α7B (lanes 1, 2 and 3) or with mAb CY8 (lanes 4 and 5). α7 bands were visualized by incubation with streptavidin conjugated to horseradish peroxidase and then detected by ECL as described in Materials and Methods. In lanes 1 and 2, some of the α7 migrates as a partially proteolytically cleaved fragment in the SDS-gel (Yao et al., 1996b). (B) Localization of α7 integrin in focal adhesions of cultured smooth muscle cells on laminin. PAC1 (a), R21696V9 (b), and 9E11G (c and d) SMCs were plated on laminin 1 substrates for 1 hour and then stained with polyclonal Ab 1211 specific for α7B integrin (a, b) or mAb CA5/CY8 for mouse α7 integrin (c). 9E11G cells were doubled-stained with mAb hVIN to vinculin (d). Note the strong staining for α7 in focal adhesions.

plated on laminin substrates and stained with anti-α7 polyclonal or monoclonal Abs, α7 integrins were concentrated at the cell periphery in vinculin-positive focal adhesions (Fig. 3B).

α7 integrin mediates adhesion and migration of smooth muscle cells

We next evaluated whether the α7 integrin could mediate adhesion and migration on laminin in cultured 9E11G smooth muscle cells. The 9E11G cell line was derived from retinoic acid-induced P19 embryonal carcinoma cells and has been shown to stably express smooth-muscle-specific α-actin and

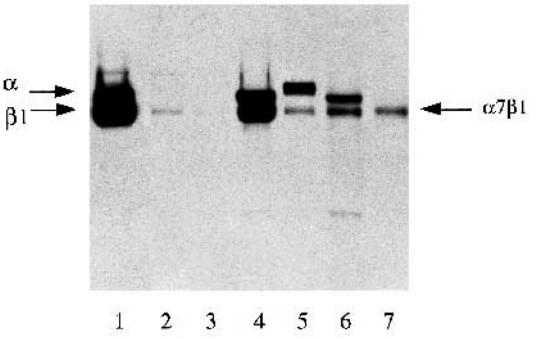

Fig. 4. Integrin profile of 9E11G smooth muscle cells. Cells were surface-biotinylated and immunoprecipitated with mAb Ha31/8 (anti-α1, lane 2), mAb Ha1/29 (anti-α2, lane 3), polyclonal Ab anti-α3 (lane 4), mAb IIA1 (anti-α5, lane 5), mAb GoH3 (anti-α6, lane 6), mAb CY8 (anti-α7, lane 7), or rabbit pAb anti-β1 (anti-β1, lane 1). Protein bands were visualized as in Fig. 3. α and β chains are indicated by arrows.

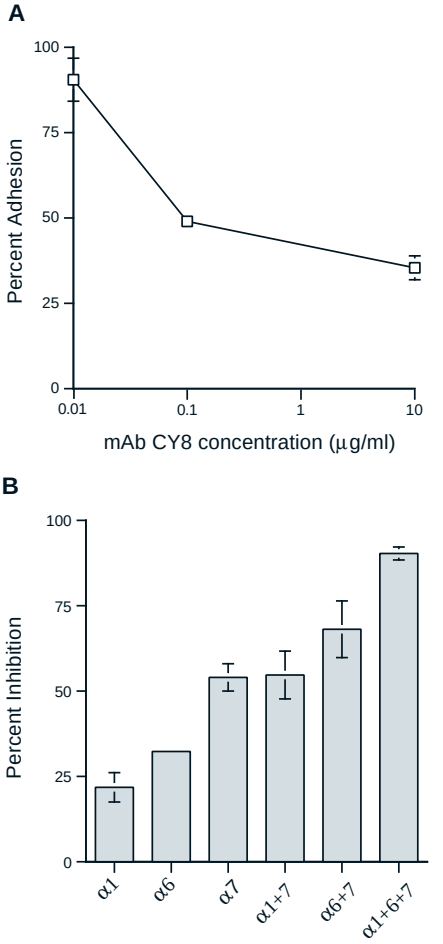

Fig. 5. α7 integrin mediates adhesion of 9E11G smooth muscle cells to laminin 1. (A) The adhesion of 9E11G cells to laminin 1 (10 μg coating) was significantly blocked in a dose-response manner with anti-α7 mAb (CY8). (B) Adhesion of 9E11G cells on laminin 1 at 10 μg/ml coating concentration was partially blocked by the presence of mAb H31/8 (anti-α1), mAb GoH3 (anti-α6) or mAb CY8 (anti-α7). The combination of anti-α1, anti-α6 and anti-α7 mAbs completely blocked adhesion of 9E11G on laminin 1. Values are means of three wells. Bars show s.d.

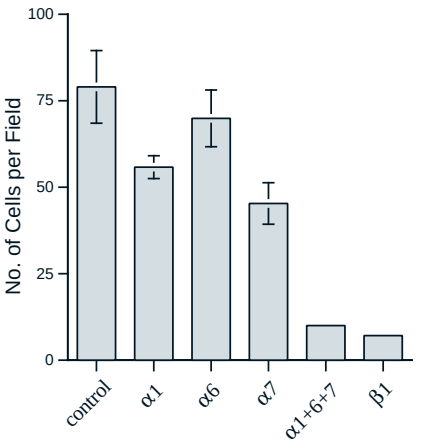

Fig. 6. α7 integrin mediates migration of 9E11G smooth muscle cells on laminin 1. Modified Boyden chamber assays were used to assess the migration of 9E11G cells as described in Materials and Methods. Control wells contained cells only without antibodies. Migration of 9E11G cells on laminin 1 was partially blocked with mAb H1/29 (anti-α1), mAb GoH3 (anti-α6) or mAb CY8 (anti-α7). The combination of anti-α1, anti-α6 and anti-α7 mAbs completely blocked migration of 9E11G cells on laminin 1, as did anti-β1 mAb (Ha2/11). The results are averages of at least nine random ×400 microscopic fields. Bars show s.d.

myosin heavy chain and to respond to contractile agonists, including PDGF-AA and PDGF-BB (Blank et al., 1995). First, we analyzed the expression of integrins on 9E11G cells by performing surface-biotinylation and immunoprecipitation; the results showed that, in addition to α7, significant levels of α1, α3, α5, and α6 chains were expressed along with high levels of the β1 subunit (Fig. 4). However, no α2 integrin was detected. The relative expression levels of this group of integrin subunits were assessed more accurately by flow cytometry using available mAbs. The mean fluorescence intensities for α1, α2, α5, α6 and α7 subunits were 5.8, 0.6, 143.9, 8.6 and 13.3, respectively. Of this set of integrins detected in 9E11G cells, α1, α3, α6 and α7 can potentially bind to laminins (reviewed by Ziober et al., 1996). We then used function-perturbing mAbs to α1 (Ha31/8), α6 (GoH3) and α7 (CY8) to assess the contribution of these integrins to mediating adhesion (Fig. 5) and migration (Fig. 6) on laminin 1; function-blocking mAb to mouse α3 integrin is not available. At 10 μg/ml of laminin coating concentration, adhesion of 9E11G cells was blocked by mAb CY8 to α7 integrin by more than 60% in a dose-response manner (Fig. 5A). However, antibodies to either α1 or α6 integrin produced only minimal blockage of 10-15% of adhesion (Fig. 5B). As expected, anti-α2 mAb Ha1/29 had no effect on adhesion to laminin (data not shown). When a combination of mAbs (anti-α1, -α6 and -α7) or anti-β1 (Ha2/11) (data not shown) was used, the adhesion of 9E11G cells on laminin 1 was almost completely blocked (Fig. 5B).

The role of α7 integrin in cell motility was assessed using the modified Boyden chamber assay with laminin 1-coated filters (Fig. 6). The migration of 9E11G cells on laminin 1 was blocked significantly with mAb to α7 (CY8), somewhat less with anti-α1 (HA31/8) and to a minor degree with anti-α6 (GoH3). As in the adhesion assay, the combination of the three mAbs (anti-α1, -α6 and -α7) was completely effective in blocking migration, as was anti-β1 mAb (Ha2/11).

Differentiating smooth muscle cells express α7 integrin

The clonal 9E11G cell line, which expresses high levels of α7, is derived from retinoic acid-treated P19 multipotential embryonal carcinoma cells. Flow cytometry analysis with mAb CY8 showed that there was no detectable α7 on the parental P19 cells (data not shown); however, after retinoic acid treatment, 30-70% of the population became positive for α7 expression (Fig. 7A). The higher the concentration of retinoic

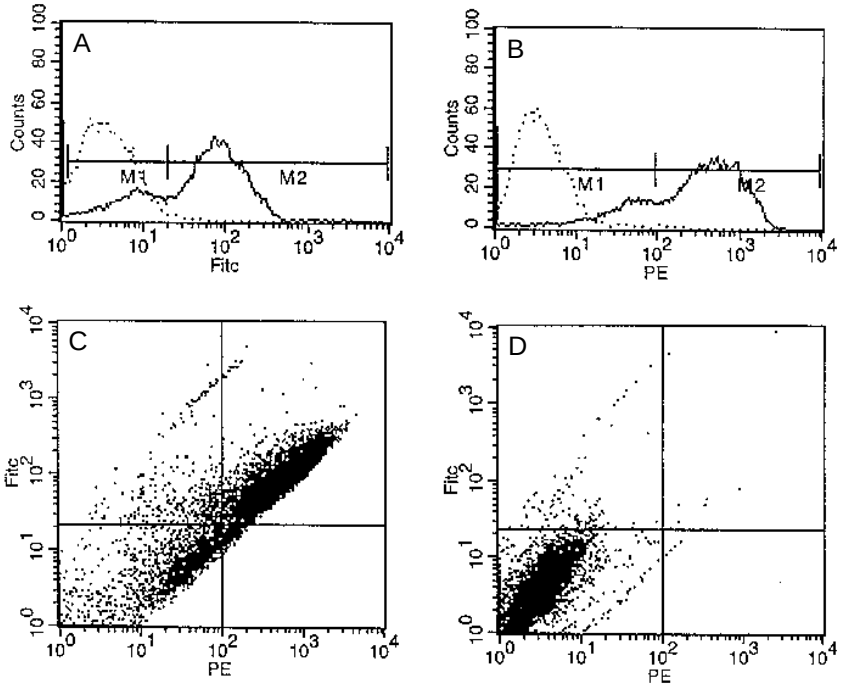

Fig. 7. Flow cytometry analysis of α7 expression in differentiating P19 cells. One week after mouse P19 embryonal carcinoma cells were treated for 48 hours with 2 μM retinoic acid, cells were resuspended and stained with (A) optimal concentrations of anti-α7 mAb (CY8), followed by incubation with FITC-labeled goat anti-rat IgG. Cells were then permeabilized with detergent and doubled-stained with (B) anti-α smooth muscle actin (α SA) (1A4) and R-phycoerythrin (PE)-labeled goat anti-mouse IgG. (C) Note that the majority of α7 positive cells were also positive for α smooth muscle actin (upper right quadrant; α SA+, α7+) whereas there were few cells in the upper left (α SA–, α7+) or lower right (α SA+, α7–) quadrant. In D, no primary antibodies were used. Two separate RA induction experiments gave similar results.

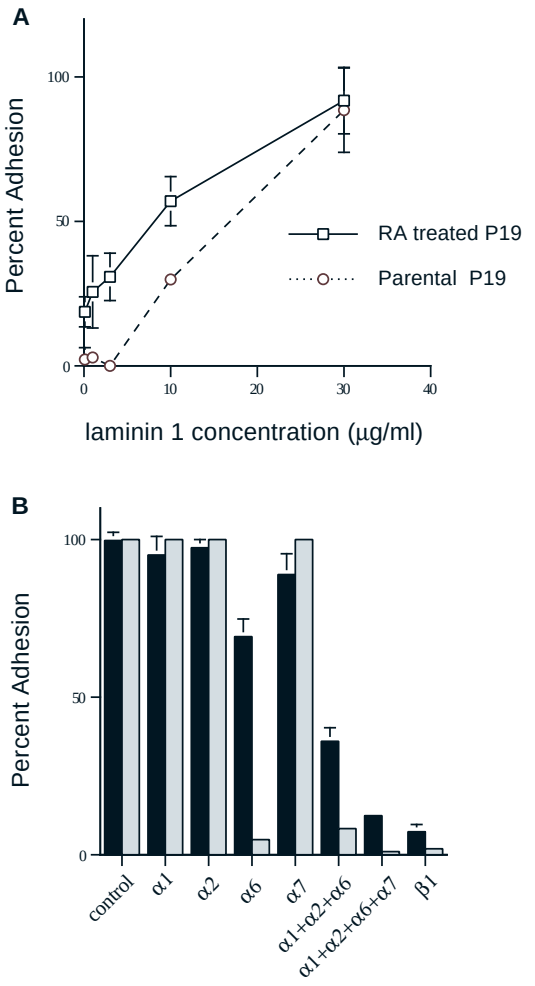

Fig. 8. α7 integrin mediates adhesion of differentiating P19 cells. (A) Adhesion of P19 cells to laminin 1 was significantly increased after retinoic acid induced cells to differentiate, especially at low laminin coating concentrations. (B) Adhesion of parental P19 cells on laminin 1 at 10 μg/ml coating concentration was completely blocked by the presence of mAb GoH3 (anti-α6) as well as by mAb Ha2/11 (anti-β1). However, at the same coating concentration, mAb GoH3 only partially inhibited the adhesion of differentiating P19 cells. Complete blocking of adhesion of this mixed population of cells to laminin 1 could be demonstrated by the combination of anti-α1, anti-α2, anti-α6 and anti-α7 mAbs as well as by anti-β1 mAb (Ha2/11).

acid, the greater the percentage of cells positive for α7 expression. To further verify whether cells expressing the α7 integrin in this differentiating population also express α-smooth muscle actin, cells were simultaneously analyzed by flow cytometry with mAb CY8 (anti-α7) (Fig. 7A) and mAb 1A4 (anti-α smooth muscle actin) (Fig. 7B). Interestingly, expression of α7 correlated positively with expression of α-smooth muscle actin; cells positive only for α-smooth muscle actin or only for α7 integrin were rarely found (Fig. 7C). This suggests that upon retinoic acid-induced differentiation, cells with α7 integrin are restricted to lineages expressing α-smooth muscle actin.

This retinoic acid-treated differentiating population of P19 cells and the undifferentiated parental P19 cells were compared for adhesion on laminin 1 substrates. Adhesion on laminin 1 significantly increased after cells were induced to differentiate (Fig. 8A). This increased adhesion appeared to be mediated in part by newly expressed α7 integrin in this differentiating population (Fig. 8B). Because mAb GoH3 could completely inhibit adhesion of parental P19 cells but not the differentiating population on laminin 1, this implied that α7 and probably α1 and/or α2 integrins expressed in this differentiating population were mediating adhesion on laminin substrates. Indeed, only when mAb CY8 was added into the combination of other function-perturbing Abs (anti-α1+α2+α6+α7), could complete inhibition of adhesion be achieved.

Downregulation of α7 integrin expression by cultured aortic SMCs

We next analyzed the expression of primary vascular SMC from the mouse aorta. In vivo, α7 integrin is expressed by aortic and coronary vascular SMCs at high levels (Fig. 9A,B). Primary aortic SMCs were prepared from explant mouse aortic segments on laminin-coated substrates. Long-term cultures were established approximately two or four weeks following explanting. Cell lysates were then prepared from surface-biotinylated cell cultures and subjected to immunoprecipitation. Surprisingly, α7 was not detected on the surface of these cultured aortic SMCs (Fig. 10, lanes 1 and 2), although β1 integrin was still readily labeled and immunoprecipitated from the same cultured cells (Fig. 10, lanes 3 and 4). However, when even a tenth of the amount of the aortic SMC lysate was processed for western blotting, a strong signal for the α7 integrin was evident (Fig. 10, lanes 5 and 6). This suggests that there is loss of α7 surface expression in aortic SMCs following prolonged in vitro cultivation. In contrast, immunostaining of cryostat sections of descending aorta showed strong surface expression of α7 integrin in the smooth muscle cell layers (Fig. 9A). A more detailed immunostaining study of aortic SMC outgrowths cultured for one week detected strong surface expression of α7 integrin on non-permeabilized cells near the edge of the explant (Fig. 11A). Interestingly, more distal from the explants, nearly all cells, which still stained positive for α-smooth muscle actin (data not shown), stained negatively for α7 integrin on cell surfaces (Fig. 11B). However, when these outmigrated α7-negative cells were permeabilized and then stained, they displayed intracellular vesicles that stained strongly for α7 (Fig. 11D). These results are consistent with a complete downregulation of α7 integrin surface expression in dedifferentiated SMCs, which may reflect failure of α7 to be exported to the surface, or alternatively, its rapid internalization.

DISCUSSION

In this study we have definitively shown that the α7 integrin is expressed at high levels in smooth muscle cells from a number of different tissues. Thus, α7 integrin is truly a muscle-specific integrin, being expressed by all major types of muscle tissue, including skeletal (Song et al., 1992; Ziober et al., 1993; Martin et al., 1996) and cardiac (Belkin et al., 1996; Collo et al., 1993; Ziober et al., 1993) as well as smooth muscle. Although α7 may also be present to some degree in peripheral

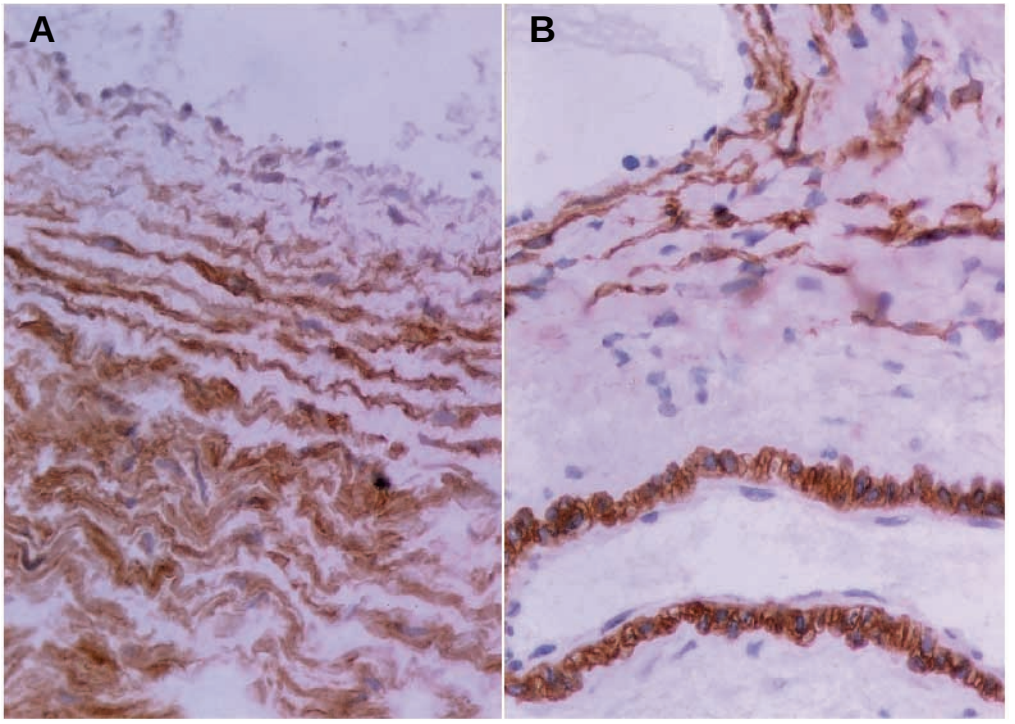

Fig. 9. Expression of α7 integrin in vascular SMCs. Frozen sections of mouse descending aorta (A) and coronary artery and vein (B) were incubated with mAbs CA5 and CY8, followed by avidin-biotin-peroxidase complex staining methods as described in Materials and Methods. α7 integrin was detected intensely at the SMC surface in the media layer.

nerve and melanocytic cells (Kramer et al., 1989; Martin et al., 1996; Sung and Bronner-Fraser, 1996), it is clear that this integrin has a highly tissue-specific and limited expression pattern. Immunohistochemical staining of developing mouse and human fetal SMCs revealed that expression of α7 occurs as a late event (data not shown). Its presence in all muscle types suggests a role for the integrin in transducing myofilament-generated forces to anchoring sites in the surrounding laminin-rich basement membrane during cellular contractile activity.

α7β1 binds laminins (Echtermeyer et al., 1996; Kramer et al., 1989; Yao et al., 1996a,b), although one report indicated that this integrin can also bind fibronectin (Gu et al., 1994). In vivo, human SMCs are surrounded by a basement membrane that contains laminin 1 (α1β1γ1) and/or laminin 3 (α1β2γ1); this may vary depending on stage of development or tissue sites (Glukhova et al., 1993). However, the presence of other α chains of laminin, but not α2, in smooth muscles has been suggested (Glukhova et al., 1993; Regenass et al., 1994; Schuler and Sorokin, 1995). Since there are at least 11 different laminin isoforms (Timpl, 1996), the presence of additional laminins associated with α7 integrin in smooth muscles remains to be determined. In previous studies, α7 expressed either in skeletal myoblasts (Yao et al., 1996b) or in transfected carcinoma cells (Yao et al., 1996a) bound to laminins 1 and 2/4 but not laminin 5. The function of α7 integrin in SMCs is demonstrated by in vitro assays in which the integrin mediated both adhesion and migration of SMC on laminin 1 substrates. Our studies using blocking mAbs showed that the interaction of SMCs with laminin involves not only α7, but also α1 and α6 integrins. However, in the case of the 9E11G SMCs, α7 appeared to be the predominant receptor for laminin 1, with α1 and α6 playing a cooperative role. Smooth muscle cells commonly form dense bodies or attachment plaques that resemble hemidesmosomes found in epithelial and endothelial cells (Glukhova and Koteliansky, 1995; Small and North, 1995). That α7 is enriched in these structures is suggested by the punctate staining of SMCs in vivo (Fig. 2) and the concentration of α7 in focal contacts on laminin substrates (Fig. 3B).

The expression of α7 by SMCs appears to correlate with the level of smooth muscle differentiation. This is shown in the current study where retinoic acid induction of multipotential stem cells produced concurrent expression of α-smooth muscle actin and α7 integrin. When α7-negative multipotential

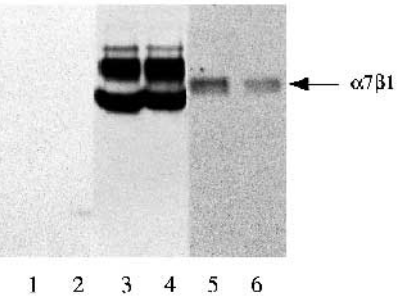

Fig. 10. Downregulation of cell-surface expression of α7 integrin in cultured aortic smooth muscle cells. SMCs from explants of mouse descending aorta were cultured for 2 or 4 weeks. Cell lysates were processed for immunoprecipitation after cell-surface biotinylation (lanes 1, 2, 3 and 4) or for western blotting (lanes 5 and 6). Immunoprecipitates are from 300 μg protein lysates from 2-week (lanes 1 and 3) or 4-week cultures (lanes 2 and 4), using anti-α7 mAb (CY8) (lanes 1 and 2) or anti-β1 polyclonal Ab 22778 (lanes 3 and 4). Samples were run on 7.5% gels, transferred to Immobilon-P membranes and visualized as described in Materials and Methods. SMCs cultured for 2 or 4 weeks showed surface expression of β1 integrins but not α7 integrin. The SMC lysates (30 μg/lane) from 2-week (lane 5) or 4-week (lane 6) cultures were processed for western blot analysis with pAb 1211 against α7B. Strong signals for α7 integrin were detected in the cell lysates.

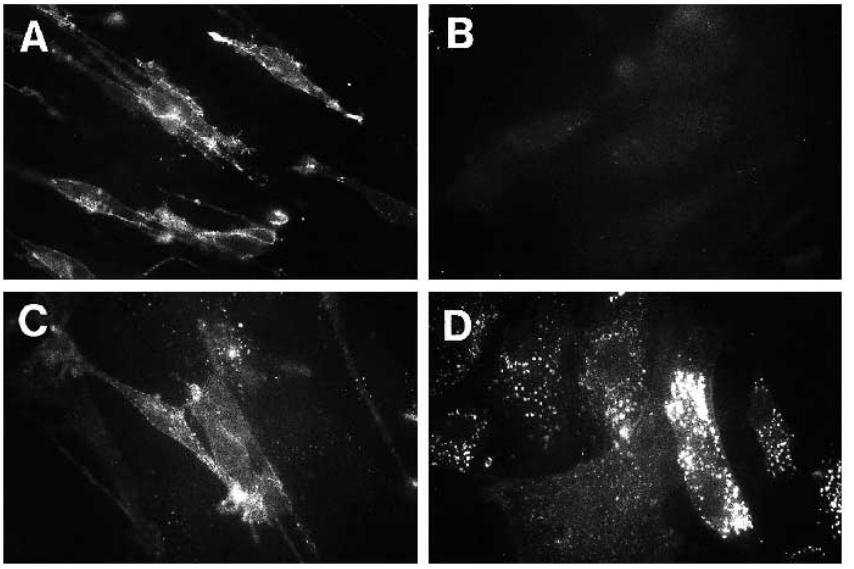

Fig. 11. Evidence for loss of surface expression of α7 integrin in aortic SMC outgrowths. Explants of descending aorta were cultured on laminin-coated coverslips for one week and then stained with mAbs CA5 and CY8 followed by FITC-labeled goat anti-rat IgG. (A,C) Regions near the edge of explant. (B,D) Peripheral regions of the migrating cell layer. Cultures were stained with (C,D) or without (A,B) 0.4% Triton X-100 detergent permeabilization. Cells near the center region expressed α7 integrin at the cell surface (A,C); cells at the peripheral region stained for α7 only intracellularly in vesicles (D).

embryonal stem cells (P19) are treated with retinoic acid, a significant proportion of the cell population differentiates along the muscle lineage (Blank et al., 1995; Rudnicki et al., 1990), which is correlated with the expression of α7 integrin. The specific phenotype of the α7-expressing subpopulation in the retinoic acid-treated P19 cells is not known because expression of α-smooth muscle actin has also been detected in the early stages of cardiac and skeletal muscle myogenesis as well as in some myofibroblastic cell lines (Lazard et al., 1993; McHugh, 1995). However, we found a correlation between expression of α7 integrin and α-smooth muscle actin. This suggests that the α7-positive cells have differentiated into the early muscle lineages. Indeed, cell clones derived from induction by these high concentrations of retinoic acid are frequently smooth-muscle-like cells (Suzuki et al., 1996).

Conversely, we show that as primary mouse SMCs dedifferentiate, following long-term adaptation to culture, and switch from the contractile to the synthetic phenotype (Hedin et al., 1988; Owens, 1995; Thyberg et al., 1990), they lose α7 expression. Similarly, our study of a number of SMC lines from different species indicated that frequently the expression of α7 is lost following in vitro culture. Decreased α1 and increased α2 integrin expression in cultured human vascular SMCs have also been shown to correlate with the transition from a contractile to a synthetic phenotype (Skinner et al., 1994).

The dedifferentiation of SMCs following adaptation to culture is a predictable and common consequence that has been well studied in the case of vascular SMCs (Owens, 1995; Thyberg et al., 1990). For example, aortic SMCs went through the modulation from a contractile into a synthetic phenotype probably because of the cells' production and deposition of fibronectin, which promotes the synthetic phenotype (Hedin et al., 1988; Thyberg and Hultgardh-Nilsson, 1994). However, certain clonal SMC lines that we screened by flow cytometry or immunoprecipitation (PAC1, R21969V9 and 9E11G) still expressed high levels of α7, and these three cell lines, like their in vivo counterparts, have been shown to stably express multiple SMC-specific differentiation markers.

One central cellular feature of atherogenesis is the accumulation and proliferation of SMCs in the tunica intima of arteries, which often involve the dedifferentiation of SMC (Raines and Ross, 1993; Ross, 1995). In the atherogenic lesion, SMCs accumulate as a consequence of a combination of directed migration and proliferation of cells from the media to the intima. In ApoB transgenic mice fed with a high-fat diet (Purcell-Huanh et al., 1995), SMCs in atherosclerotic lesions were found to have downregulated levels of α7 integrin (unpublished observation). It is likely that during the initial stages of atherogenesis, highly differentiated SMCs, which were originally arranged in concentric layers and encircled by basement membranes, become motile and migrate into the intima toward growth factor signals via unaltered integrin receptors. Besides stimulating migration, growth factors could also increase cell proliferation and modulate SMC phenotypes and integrin expression. It is possible that, following SMC outmigration using laminin-binding α7 integrin, growth factors and extracellular matrix promote the dedifferentiation and switching of SMCs to a synthetic phenotype and this, combined with other changes, eventually leads to formation of intimal plaques.

In summary, this report establishes that α7 integrin is expressed in highly differentiated SMCs both in vivo and in culture. Furthermore, the results show that during RA-induced stem cell differentiation to SMC, the expression of α7 parallels the induction of the SMC-specific marker smooth muscle α-actin. While SMCs express multiple receptors for laminin, we show here that the α7 receptor is an important mediator of adhesion and locomotion on laminin substrates. Differentiation is regulated by extracellular matrix contact in many cell types, including skeletal myoblasts (Volk et al., 1990) and SMCs (Thyberg, 1996). The α7 integrin may be important for maintenance of the SMC differentiated phenotype. At present, we know little about the regulation of α7 expression in SMCs. However, recently we have isolated and characterized the gene promoter for α7 and showed that in the case of skeletal myoblasts the α7 promoter was *trans*-activated by myogenin and MyoD but not by myf5 (Ziober and Kramer, 1996). Little information is available concerning the identity of transcription factors that regulate SMC-specific genes in differentiated cells but it is likely that 'master' regulatory transcription

factors analogous to MyoD exist in SMCs and coordinate α7 expression. Studies to identify such factors are currently underway.

We thank Dr Sanes for help with immunofluorescence staining, Dr Owens and Dr Rothman for the generous gifts of cell lines and all the contributors of antibodies and other materials. We are also grateful to Evangeline Leash for editing this manuscript. This work was supported by NIH grants R01 CA 33834, R01 DE 10306 and DE 10564 to Dr Randall Kramer.

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(Received 26 February 1997 – Accepted 25 April 1997)