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Restoration to a Quiescent and Contractile Phenotype from a Proliferative Phenotype of Myofibroblast-Like Human Aortic Smooth Muscle Cells by Culture on Type IV Collagen Gels[1]

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Aortic smooth muscle cells (A-SMC) undergo phenotypic transition to a synthetic and proliferative state and become fibroblast-like cells upon serial passage with culture on plastic dishes, especially in the presence of serum. Such fibroblast-like cells (M-SMC) derived from A-SMC may correspond to the cells identified pathologically as myofibroblasts. We examined the effects of type IV collagen gels used as a culture substrate on the morphology and proliferation of M-SMC. The M-SMC underwent extreme elongation in shape when cultured on rigid type IV collagen gels, and eventually formed cell-to-cell junctions with the elongated processes. In contrast, M-SMC showed a spindle-like cell shape on dishes coated with a type IV collagen solution or type I collagen solution, or on type I collagen gels or fragile type IV collagen gels. Cell proliferation was totally repressed by culture on rigid type IV collagen gels for over 10 days, while the highest proliferative activity was seen for cells grown on dishes coated with type IV collagen solution. The expression of smooth muscle myosin heavy chains, specific markers for contractile A-SMC, was acquired by M-SMC cultured on rigid type IV collagen gels for 3 days, while M-SMC cultured on type IV collagen-coated dishes continued to show no expression. These results suggest that the quiescent and contractile phenotype of A-SMC might be restored in M-SMC by culture on rigid type IV collagen gels, even after they have become myofibroblastic.

Key words: aortic smooth muscle cell, cell proliferation, gel culture, myofibroblast, type IV collagen.

It has been reported that in atherosclerotic lesions aortic smooth muscle cells (A-SMC) change their differentiation state from contractile to synthetic (*1, 2*). A-SMC phenotypes that change within the lesions include morphological appearance, proliferative activity, production of fibrillar collagen, and the deposition of collagen fibrils (*3, 4*), all of which are similar to features of fibroblasts. These observations have tempted pathologists to refer to smooth muscle-derived cells as myofibroblasts. The regulation of smooth muscle phenotypes is considered to be crucial in the pathogenesis of fibrosis or cirrhosis of various organs. A similar change to a fibroblastic phenotype occurs in A-SMC during prolonged periods of primary culture *in vitro* and/or repeated passages in serum-containing culture medium (*3, 5-7*). These cells may have acquired properties corresponding to those of the myofibroblasts found in atherosclerotic lesions (*3, 8*).

The fundamental functions of cells, including growth, maintenance of differentiation, gene expression, cell migration, as well as cell attachment, are affected by the surrounding extracellular matrix (ECM). Direct evidence has been obtained by *in vitro* studies that have analyzed the effects of substrates on cultured cells (*9-12*). The functions of A-SMC are affected and thus regulated in cell culture by ECM used as consolidated culture substrates (*3, 13*). Previous reports describe how laminin and type IV collagen might help to retard the de-differentiation of cultured A-SMC during an initial period without serum (*14, 15*). However, A-SMC *in vitro* tend to lose their differentiated phenotypes during longer terms in culture. Particularly in the presence of serum, A-SMC become fibroblastic, regardless of whether they are cultured on dishes coated with any of the ECM components examined previously. Solubilized collagenous proteins are not exceptional (*13, 16*).

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Abbreviations: α-SMA, α-smooth muscle actin; A-SMC, aortic smooth muscle cell; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HF, human dermal fibroblast; M-SMC, myofibroblast-like smooth muscle cell; PBS(−), Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline; PDL, population doubling level; SMH, smooth muscle myosin heavy chains.

Isolated type I collagen can be reconstituted into fibrillar aggregates with a characteristic banding pattern. Upon fibril formation, the type I collagen solution becomes a gel under physiological conditions of pH, salt concentration, and temperature. When A-SMC that have become fibroblast-like in culture are cultured within reconstituted type I collagen gels, their phenotypes are quite similar to those of fibroblasts (*17*); that is, the collagen gel is contracted by these cells (*17-20*). The cell shape is extremely elongated, while cell proliferation is repressed (*18, 21, 22*). Recently it was reported that fibrillar type I collagen (*23*) or the gel forms of type I and type III collagen (*18*) retard A-SMC proliferation in primary cultures without serum. These findings prompted us to speculate that assembled collagenous proteins might show different activities from those of monomeric or soluble proteins.

We have hypothesized that tissue collagen type distribution shows a graded structure (*24*). We have recently extended this idea so that the localization of cell types is incorporated into the tissue graded structure of collagen aggregates, especially the tissue co-localization of type IV collagen and A-SMC. That is, A-SMC are in contact with type IV collagen in tissue, while dermal fibroblasts are not (*25*). We then addressed the question of whether the relationships between collagen aggregates and the corresponding cell types in tissues have biological meaning such as the maintenance of cell phenotypic differentiation.

This hypothesis was tested by asking whether smooth muscle cell-derived fibroblast-like cells (myofibroblasts in short) and dermal fibroblasts are affected differently by the substrate of reconstituted type IV collagen aggregates, since we recently found that isolated type IV collagen forms a polygonal meshwork with pores 18 nm in diameter corresponding to the skeletal structure of the lamina densa (*26*). Upon formation of the polygonal meshwork, the type IV collagen solution gels under selected conditions of NaCl concentration and temperature (*27*). Gel rigidity depends especially on temperature and incubation time as well as on protein concentration (*27*). Contrary to the case of type I collagen gels, the rigidity of type IV collagen gels requires a low temperature. We here report the effects of a rigid type IV collagen gel used as a cell culture substrate on the fundamental cellular functions of myofibroblast-like A-SMC (designated M-SMC in this report), including cell shape, cell growth, and marker protein expression in cultures with 10% FBS.

EXPERIMENTAL PROCEDURES

Preparation of Lens Capsule Type IV Collagen—Bovine lens capsules were kindly provided by Nitta Gelatin, Osaka; otherwise they were isolated from bovine eyes purchased from Tokyo Shibaura Zoki, Tokyo. All the following procedures were carried out at 4°C. Type IV collagen was extracted with acid from bovine lens capsules without pepsin treatment as previously described (*28*). Briefly, lens capsules were homogenized with a Polytron homogenizer in 0.5 M acetic acid (5-10 ml per 1 g wet weight of lens capsule) containing a mixture of protease inhibitors, 5 mM EDTA, 10 mM *N*-ethylmaleimide, 100 μM phenylmethylsulfonyl fluoride, 1 mM pepstatin A, and 0.02% sodium azide. The homogenates were stirred in suspension for 2-3 days. After centrifugation at 800 × *g* for 15 min, the supernatant was collected and precipitated by the addition of 1.2 M NaCl. The precipitate was dissolved in 0.5 M acetic acid and the solution was dialyzed extensively against 1 mM HCl and stored at 4°C before use. Protein concentrations were determined by the weight of the lyophilized material.

Cell Culture—Human A-SMC at passage 3 were purchased from Clonetics (San Diego, CA, USA). The cells were cultured in modified MCDB131 medium (Clonetics) supplemented with 10% FBS (Cansera International, Canada), 10 ng/ml recombinant epidermal growth factor, 2 ng/ml recombinant basic fibroblast growth factor, 5 μg/ml insulin, 50 μg/ml gentamicin, and 50 ng/ml amphotericin-B (designated as Growth medium) at 37°C under an atmosphere of humidified 5% CO_2-95% air. Growth factors, insulin, and antibiotics were purchased from Clonetics. Normal human dermal fibroblasts, HF-18, were a generous gift from Dr. Toshio Nishiyama of the Shiseido Research Center, Yokohama, and cultured in DMEM (Nissui Pharmaceutical, Tokyo) containing 10% FBS, 60 μg/ml kanamycin sulfate, and 50 ng/ml amphotericin-B at 37°C. The cells were routinely maintained on 100 mm tissue culture dishes (Falcon, No. 3003). The cells at confluence were passaged at a 1:4 split ratio after removal from the dish by brief exposure to 0.25% trypsin-0.02% EDTA in PBS(−). M-SMC obtained from A-SMC by 3-5 passages up to 9-13 PDL on 100 mm dishes were cultured on different substrates.

Preparation of Substrates and Successive Cell Culture—Type I collagen was obtained as an acid extract of rat tail tendon by a previously reported method (*29*). Preparation and successive culture of the cells on type I collagen gel were performed as previously described (*29*). Briefly, 6 volumes of type I collagen solution (3 mg total protein/ml) was mixed with 3 volumes of 3×concentrated Growth medium without FBS or 3×concentrated DMEM for HF-18 and 1 volume of FBS at 4°C to give a final collagen concentration of 1 mg/ml. Aliquots (500 μl) of the solution were added to each well of 24-well tissue culture dishes (Falcon, No. 3047) and incubated at 37°C for gelation. One milliliter of cell suspension containing 7.2×10^5 cells was plated on each gel and the cells were grown at 37°C under 5% CO_2-95% air. The medium was replaced every 3 days. The preparation of type IV collagen gels has been described previously (*27*). In brief, 9 volumes of type IV collagen solution (2 mg/ml) in 1 mM HCl was mixed with 1 volume of 200 mM phosphate buffer containing 1.5 M NaCl, pH 7.3, at 4°C to obtain a final collagen concentration of 1.8 mg/ml. Aliquots (500 μl) of the solution were added to each well of 24-well Falcon tissue culture dishes. The dishes were incubated at 4°C for at least 5 days for gel formation to provide sufficient rigidity to accommodate cells on top of the gel. After replacing the buffer with Growth medium or DMEM containing 10% FBS for HF-18, 1 ml of cell suspension containing 7.2×10^3 cells was gently placed on each gel and the cells were cultured at 37°C under 5% CO_2-95% air. The medium was replaced every 3 days. Type IV collagen gels with insufficient rigidity were also used to test whether gel rigidity affects cellular behavior. Aggregated type IV collagen-coated dishes were prepared as follows. Nine volumes of type IV collagen solution (2 mg/ml) in 1 mM HCl was mixed with 1 volume of 200 mM phosphate buffer containing 1.5 M NaCl, pH 7.3, to give a final collagen

concentration of 1.8 mg/ml. The neutralized solution was added to 24-well dishes (500 μl/well) and then dried at 25 C. The wells were washed with PBS(−), followed by further washing with Growth medium. For the preparation of protein-coated dishes, type I or type IV collagen solution in 1 mM HCl (100 μg/ml) was placed in 24-well dishes (250 μl/well) and allowed to adsorb to the dish surface at 37 C for 2 h. The wells were then washed with PBS(−), followed by further washing with either Growth medium (for M-SMC) or DMEM containing 10% FBS (for HF-18). Cell morphology was observed with a phase-contrast microscope (Model DMIRB, Leica, Tokyo) at 100× magnification.

Cell Proliferation Assay—The quantification of cell proliferation was performed as previously described (*30*). In brief, cells cultured on type I collagen and type IV collagen gels were washed with PBS(−) and then treated with 500 μl of 0.2% bacterial collagenase (Wako Pure Chemicals, Tokyo) containing 1 mM $CaCl_2$ in PBS(−) at 37 C for 2 h to suspend the cells. Cells cultured on plastic

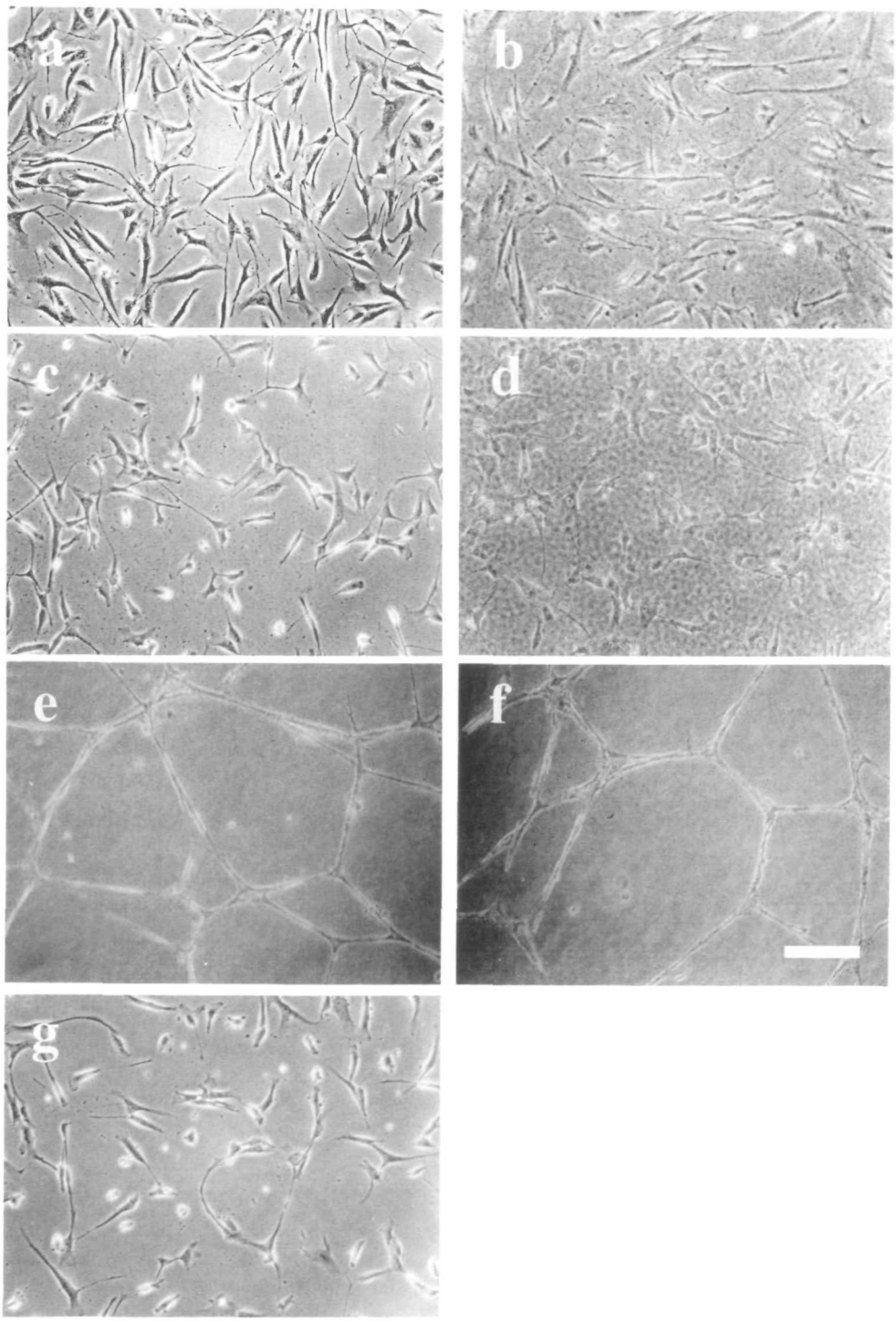

Fig. 1. **Morphology of M-SMC cultured on different substrates.** M-SMC obtained from A-SMC by repeated passages up to 9 PDL were cultured on type IV collagen-coated dishes (a), type I collagen gels (b), aggregated type IV collagen coated dishes (c), type IV collagen gels with insufficient rigidity (d), or type IV collagen gels with sufficient rigidity (e, f). The cells grown on rigid type IV collagen gels at culture day 10 were liberated by treatment with 0.2% bacterial collagenase and re-cultivated on plastic dishes for 3 days (g). At culture day 3 (a-e, g) or day 14 (f), the morphology of the M-SMC was observed by phase-contrast microscopy and photomicrographed. Bar: 100 μm.

dishes or dishes coated with type I collagen solution or type IV collagen solution were removed with 500 μl of 0.25% trypsin-0.02% EDTA in PBS(−). The numbers of cells liberated were counted in triplicate wells on day 2, 5, and 10 with a Coulter counter (Model Z-1, Coulter, Tokyo).

Immunocytochemistry—M-SMC cultured on dishes coated with type IV collagen solution, type I collagen gel, or rigid type IV collagen gel for 3 days or 7 days were fixed with 4% paraformaldehyde at 4°C for 18 h and permeabilized with 0.5% Triton-X100 in PBS(−) at 25°C for 90 min. The cells were then preincubated with 0.2% BSA in PBS(−) for 30 min to block nonspecific binding, and incubated with a 1:500 dilution of anti-smooth muscle myosin heavy chain monoclonal antibody (Clone hSM-V, Sigma, USA) or an anti-human β1 integrin monoclonal antibody (MAB 1977, Chemicon Int., USA) at 4°C for 18 h. After exposure to a 1:150 dilution of FITC-conjugated goat anti-mouse IgG (Leinco Technol., USA), the cells were observed under a confocal laser scanning microscope (Model TCSNT, Leica, Tokyo) in fluorescence mode at 400× magnification. Non-immune mouse IgG (ICN Pharmaceuticals, USA) was used as a control in place of specific antibodies against SMH and human β1 integrin.

Endothelin-1 Treatment—M-SMC were cultured on 24-well Falcon dishes coated with type I collagen solution and type IV collagen solution, or on type I collagen gel and rigid type IV collagen gel for 3 days in Growth medium. The cells were treated with 1 nM human endothelin-1 (Peptide Inst., Osaka) and observed with the phase-contrast microscope at 37°C for 15 min. Photomicrographs were taken every 1 min at 100× magnification. Cells without the addition of endothelin-1 were used as controls.

RESULTS

Morphology of M-SMC in Culture—In the presence of 10% FBS, repeated passages on plastic dishes of A-SMC starting from passage 3 up to 9-13 PDL caused the cells to acquire a high growth activity. In this study, we refer to these cells as myofibroblast-like smooth muscle cells or M-SMC in short. To determine whether the chemical and supramolecular structures of type I collagen and type IV collagen affect the cell morphology of M-SMC, we prepared six different substrates as follows: (i) dishes coated with type I collagen solution, (ii) dishes coated with type IV collagen solution (Fig. 1a), (iii) type I collagen gel (Fig. 1b), (iv) dishes coated with aggregated type IV collagen (non-gel form) (Fig. 1c), (v) type IV collagen gel with sufficient rigidity (Fig. 1, e and f), and (vi) type IV collagen gel with insufficient rigidity (Fig. 1d). The cell morphology of M-SMC at 9 PDL on the six different substrates was observed under a phase-contrast microscope at culture day 3. The M-SMC started to spread 2 h after cultivation on all substrates except type I collagen gel or type IV collagen gel with sufficient rigidity. Cell spreading on the type I collagen gel started at 5 h, and then showed morphology with a spindle-like or elongated bipolar cell shape similar to cells cultured on dishes coated with type IV collagen solution (Fig. 1, a and b), dishes coated with aggregated type IV collagen (Fig. 1c), bare plastic dishes, and type I collagen-coated dishes (data not shown). Culturing M-SMC on rigid type IV collagen gels caused the start of cell spreading and elongation to be retarded to as late as cells cultured on type I collagen gels. Once the cells started to elongate, the elongation proceeded for about 2 days. Thereafter, extremely elongated cell termini could not be distinguished due to the apparent formation of cell-to-cell junctions with adjacent elongated cells. Cell-to-cell junctions formed over the entire cell surface, eventually giving rise to a mesh-like multicellular organization as shown in Fig. 1e. The meshwork formation was completed by culture day 3 and maintained for more than 14 days (Fig. 1f). The multicellular meshwork structure began to collapse upon treatment with bacterial collagenase. Finally, the cells detached from one another assuming a round shape. However, when the rounded cells were collected and seeded again on the bare plastic dishes, they again assumed a spindle-like shape with high proliferative activity (Fig. 1g). These findings indicate that the repression of M-SMC proliferation on rigid type IV collagen gels is reversible.

It takes longer for type IV collagen solutions to gel to a rigidity comparable to type I collagen gels. Fragile gels were obtained when the incubation time at 4°C was shortened. Thus, when cells were placed on type IV collagen gels with insufficient rigidity, they sometimes sedimented to the bottom of the gel. Such cells in contact with the dish eventually begin to spread. Finally, they show a cell shape similar to cells cultured on dishes coated with type IV collagen solution (Fig. 1d). The results suggest that the morphology of M-SMC is strongly affected by whether the type IV collagen aggregates are in a gel form or not.

Proliferation of M-SMC in Culture—M-SMC subjected to repeated passages on plastic dishes up to 9 PDL acquire a high growth potential as shown in Fig. 2. M-SMC proliferation was determined in the presence of 10% FBS on different substrates. Cell attachment is a prerequisite for cell growth, and the numbers of cells attached to the substrate did not differ among the substrates examined.

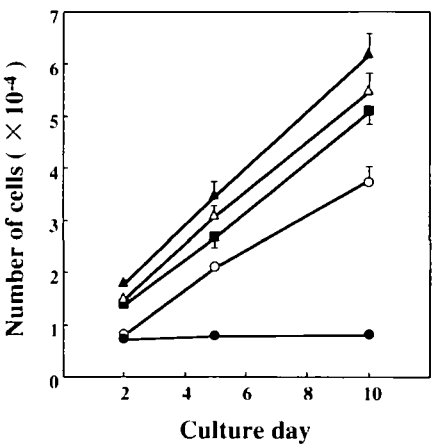

Fig. 2. **Growth curves of M-SMC cultured on different substrates.** M-SMC obtained from A-SMC through repeated passages up to 9 PDL were cultured on plastic dishes (■), type I collagen-coated dishes (△), type IV collagen-coated dishes (▲), type I collagen gels (○), or rigid type IV collagen gels (●) in Growth medium as described in "EXPERIMENTAL PROCEDURES" for 10 days. The cells were initially plated at a cell density of 7.2×10^3/well. The medium was renewed every 3 days. The cell number was counted on days 2, 5, and 10 with the Coulter counter after the cells were removed from the dishes. Each point represents the mean of three determinations with the standard deviation.

M-SMC cultured on type I collagen gels did not initiate cell growth until day 2. However, once cell growth started, the growth rate on type I collagen gels was as high as that of cells on plastic dishes (Fig. 2). The highest cell number on culture day 10 was seen for M-SMC cultured on dishes coated with the type IV collagen solution (Fig. 2). On the other hand, the number of M-SMC did not increase at all over 21 days on the rigid type IV collagen gels (Fig. 2).

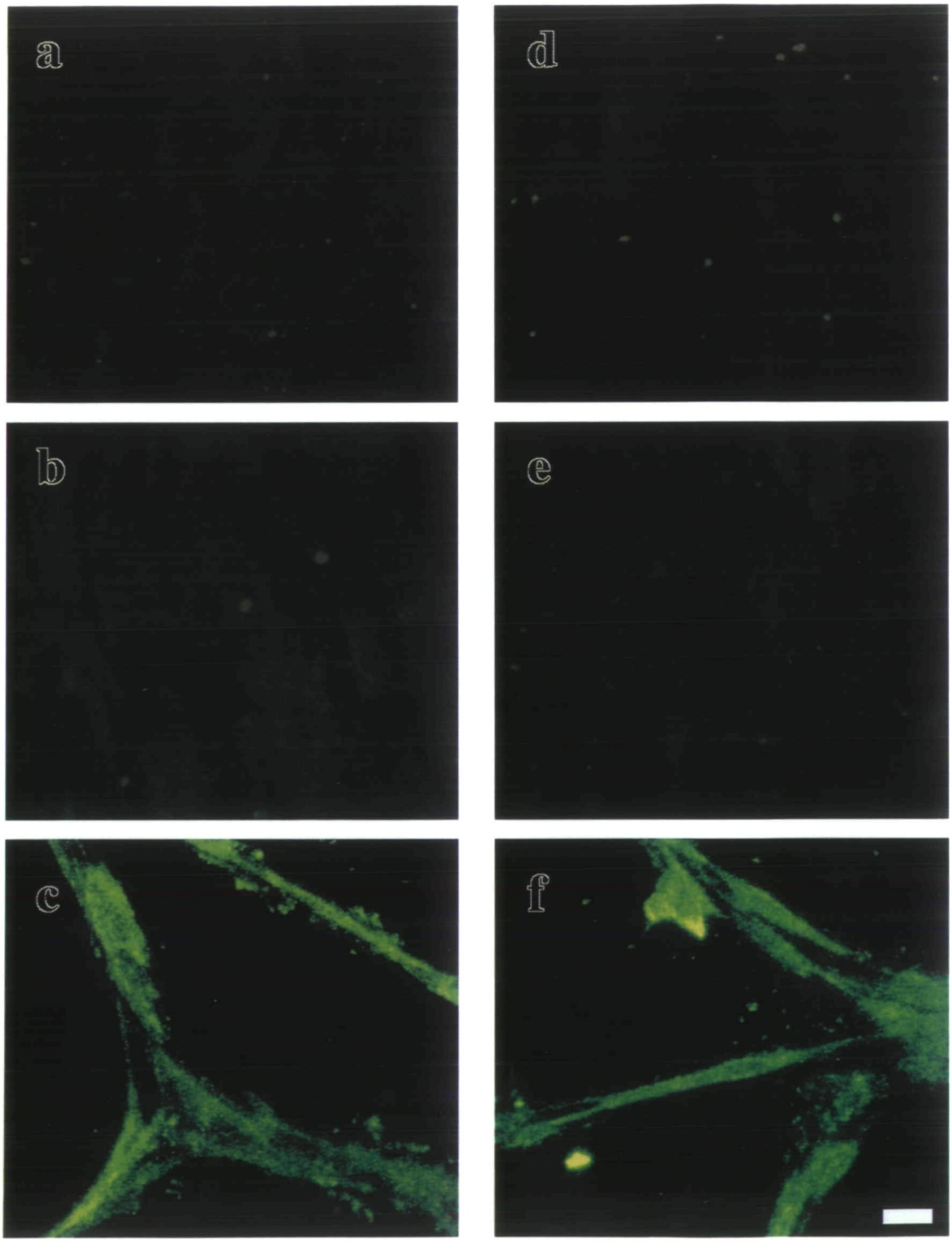

Fig. 3. **Expression of SMH in cultured M-SMC.** M-SMC obtained from A-SMC through repeated passages up to 13 PDL were cultured on type IV collagen-coated dishes (a, d), type I collagen gels (b, e), or rigid type IV collagen gels (c, f) for 3 days (a-c) or 7 days (d f). The cells were fixed and permeabilized at 25 C, and then incubated with anti-SMH monoclonal antibodies at 4 C for 18 h. After exposure to FITC-prelabeled goat anti-mouse IgG, the cells were observed by confocal laser scanning microscopy and photomicrographed. Bar: 10 μm.

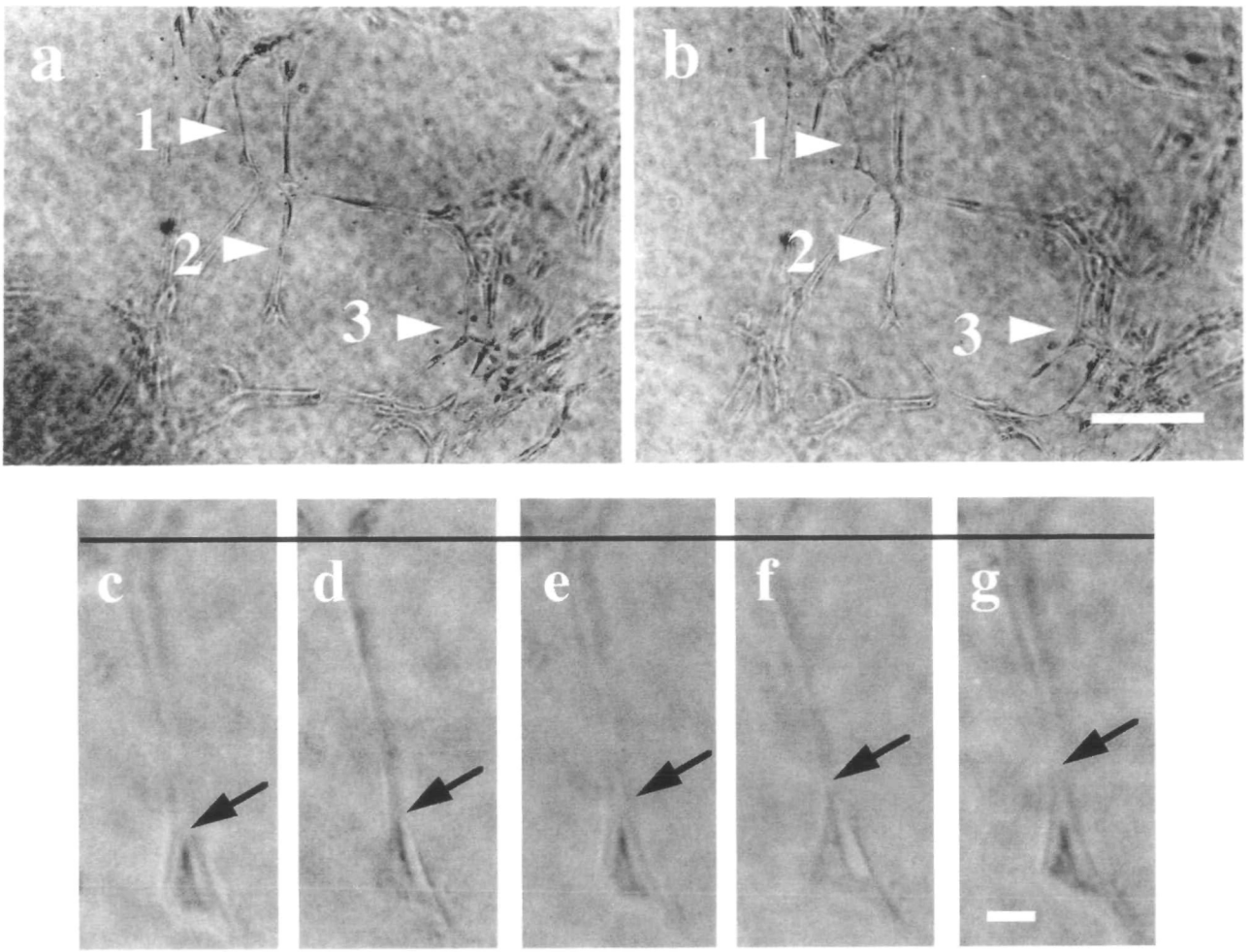

Fig. 4. **The effect of endothelin-1 on the contraction of M-SMC.** M-SMC at 13 PDL cultured on rigid type IV collagen gels were treated with 1 nM endothelin-1 on culture day 3. Cells are shown before (a) and after (b) the addition of 1 nM endothelin-1 for 15 min. Arrowheads indicate representative points noted as having moved upon the addition of endothelin-1. Shown are a cell-body bent to a bow-like shape (arrowhead 1) and extended to a thin line (arrowhead 2). Arrowhead 3 indicates that the curve formed by the two cells appears to be smoothed. Bar: 100 μm. Higher magnifications of the point indicated by the arrowheads 1 in (a) and (b) are shown in (c) (g). Photographs were taken before (c) and after the addition of 1 nM endothelin-1 at 1 min (d), 4 min (e), 10 min (f), and 15 min (g). It appears that the same region of the cell body indicated by the arrows has moved up. With the black line being taken as a reference, the contracted distance is about 15 μm at 15 min. White bar: 10 μm.

Further addition of other growth factors did not release the repression apparently exerted by the rigid type IV collagen gel (data not shown). The growth arrest is not due to apoptosis of the cells, since the quiescent M-SMC started to proliferate upon treatment with bacterial collagenase and re-cultivation on plastic dishes (Fig. 1g).

Expression of Marker Proteins for Contractile Stage A-SMC in Culture Differentiation of A-SMC is usually evaluated by examining whether the smooth muscle-specific cytoskeletal proteins, including α-smooth muscle actin (α-SMA) (*31, 32*), smooth muscle myosin heavy chains (SMH) (*33, 34*), and caldesmon (*35*), are expressed or not. Among these marker proteins, SMH is generally assumed to be the most specific and reliable marker for the contractile stage of A-SMC (*34*). For analysis, an antibody that does not cross-react with skeletal, cardiac, or non-muscle myosins is required (*36*). Such anti-SMH monoclonal antibodies were used for the immunocytochemical analysis of the expression of SMH in M-SMC at 13 PDL cultured on different substrates. SMH was strongly stained in M-SMC cultured for 3 days on rigid type IV collagen gels (Fig. 3c). On the other hand, M-SMC displayed little SMH expression when cultured on dishes coated with type IV collagen solution (Fig. 3a). SMH seemed to be expressed in cells cultured on type I collagen gels, but only slightly (Fig. 3b). At culture day 7 on type IV collagen-coated dishes, the cells showed no immunoreactivity to SMH at all (Fig. 3d). Diffuse and weak SMH staining was noted in SMC cultured on type I collagen gels (Fig. 3e). In contrast, strong immunostaining revealed the maintenance of marked SMH expression in M-SMC cultured for 7 days on type IV collagen gels with sufficient rigidity (Fig. 3f). Background fluorescence was null in the control specimens on culture days 3 and 7 treated with non-specific IgG (data not shown).

Contraction of M-SMC in Culture by the Addition of Endothelin-1 Endothelin-1 at a final concentration of 1 nM was added to M-SMC at 13 PDL on culture day 3 to see whether contractility is maintained or restored by the drug.

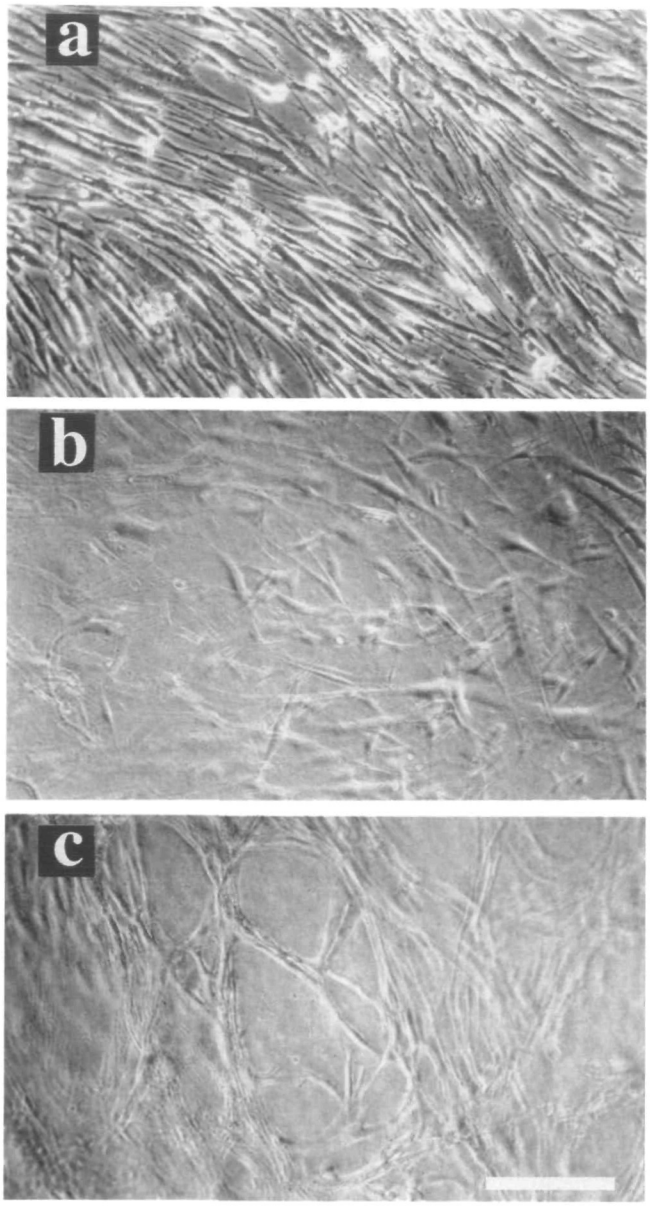

Fig. 5. **The effect of substrates on the morphology of cultured HF-18.** HF-18 were cultured on type IV collagen-coated dishes (a), type I collagen gels (b), or rigid type IV collagen gels (c). On culture day 5, the morphology of HF-18 was observed by phase-contrast microscopy and photomicrographed. Bar: 100 μm.

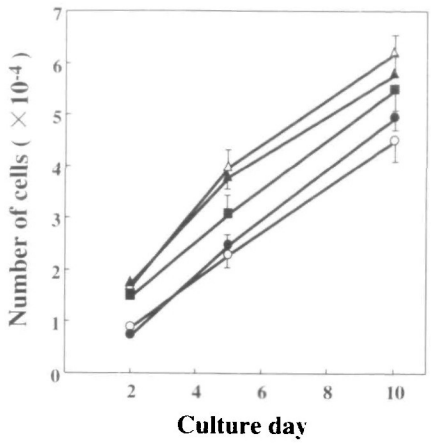

Fig. 6. **Growth curves of HF-18 cultured on different substrates.** HF-18 were cultured on plastic dishes (■), type I collagen-coated dishes (□), type IV collagen-coated dishes (▲), type I collagen gels ( ), or rigid type IV collagen gels (●) in DMEM supplemented with 10% FBS as described in "EXPERIMENTAL PROCEDURES" for 10 days. The cells were initially plated at a cell density of 7.2×10^3/well. The medium was renewed every 3 days. The number of cells was counted on days 2, 5, and 10 with a Coulter counter after the cells were removed from the dishes. Each point represents the mean of three determinations with the standard deviation.

Upon the addition of endothelin-1, only M-SMC cultured on rigid type IV collagen gels slightly and slowly changed their morphology in the multicellular network (Fig. 4). The 15 consecutive photographs taken once every minute show a slight but marked movement of portions of the cell meshwork. No further cell movements were seen after 15 min. The change in morphology may correspond to the contraction of M-SMC (Fig. 4). Contraction was not observed in cells cultured on dishes coated with type IV collagen solution and type I collagen solution or on type I collagen gels (data not shown).

Morphology of HF-18 in Culture—Cell attachment, spreading, and elongation of normal human dermal fibroblasts, HF-18, shortly after seeding on dishes coated with type IV collagen solution, type I collagen gel, or rigid type IV collagen gel were similar to those of M-SMC observed under phase-contrast microscopy. HF-18 appeared elongated or spindle shaped on all substrates. HF-18 cultured on rigid type IV collagen gels also formed multicellular meshworks at culture day 2. However, as HF-18 cells started to grow on rigid type IV collagen gels, the meshwork structure appeared to dissipate. On culture day 5, HF-18 and M-SMC showed quite different shapes on rigid type IV collagen gels as shown in Fig. 5c (Compare with Fig. 1e). HF-18 did not retain intercellular junctions on rigid type IV collagen gels. HF-18 cultured on dishes coated with type IV collagen solution (Fig. 5a) or type I collagen gels (Fig. 5b) showed similar morphologies to M-SMC cultured on these substrates.

Proliferation of HF-18 in Culture—The proliferation of cultured HF-18 was examined on different substrates. The attachment of HF-18 was the same regardless of substrate used. Although HF-18 showed a 2 day lag period before starting to grow on type I collagen gels or rigid type IV collagen gels, their growth rate was almost the same on all substrates (Fig. 6). HF-18 grew on rigid type IV collagen gels in contrast with M-SMC (Fig. 6).

DISCUSSION

It is well known that A-SMC recovered from artery tissues as primary contractile cells eventually become synthetic through repeated passages in *in vitro* cultivation, particularly in the presence of serum (*3, 5, 6, 13*). The synthetic A-SMC thus obtained are named M-SMC in this report. During the initial stages of culture (up to culture day 2), essentially no differences in morphological characteristics were seen for M-SMC or HF-18. However, morphological characteristics were noted for M-SMC cultured on rigid type IV collagen gels at day 5 that were distinct from those

of M-SMC cultured on other substrates or HF-18 cultured on rigid type IV collagen gels (Figs. 1 and 5). These characteristics can be summarized as follows: (i) highly elongated shape, (ii) side-by-side association of the elongated cells, and (iii) cell-to-cell contacts at the tips of adjacent cells, presumably with the formation of cell-to-cell junctions. The multi-cellular structure at a low cell density forms a cell network as a whole (Fig. 1, e and f). A possibly corresponding case *in vivo* can be found in small blood vessels where pericytes are rather sparsely distributed underneath endothelial cells (*37*).

Culture on dishes coated with type IV collagen solution facilitated the proliferation of M-SMC in the presence of serum (Fig. 2) in comparison with cells cultured on bare plastic dishes. To our surprise, however, the rigid type IV collagen gels had an entirely opposite effect; that is, M-SMC proliferation was repressed. M-SMC were totally quiescent up to 21 days in culture on rigid type IV collagen gels, even in the presence of 10% FBS (Fig. 2). As stated above, dishes coated with type IV collagen solution had a growth stimulating effect similar to, if not stronger than, dishes without any coating or dishes coated with type I collagen solution (Fig. 2). Correspondingly, the cell morphology was similar among M-SMC cultured on all substrates except the rigid type IV collagen gels (Fig. 1). These results indicate that the protein structure of type IV collagen alone can not be responsible for the apparent effect of rigid type IV collagen gels on the behavior of M-SMC. Instead, a type IV collagen gel with sufficient rigidity is important. The contact surface areas between the fine filaments comprising the gel and cells are restricted. Thus, most of the basal surface of the cells may be open to diffusible substances. The study of the immunocytochemical localization of β1 integrin showed a diffuse distribution over the entire surface of the basal surface regardless of the substrate (data not shown), as was seen for the diffuse or spotted localization of β1 integrin on fibroblasts cultured in type I collagen gels (*38*).

α-SMA and calponin are known protein markers for differentiated A-SMC in the contractile state (*31, 32, 39*). However, the specificity of α-SMA for the contractile state of A-SMC has recently been suspected, because the proteins are expressed in A-SMC in the synthetic state and in non-muscle cells (*40*). On the other hand, SMH seems to be specific for A-SMC in the contractile state (*33, 34*). Immunocytochemical analyses with anti-SMH antibody revealed that rigid type IV collagen gels induce the expression of SMH in M-SMC by culture day 3 (Fig. 3c) and the expression continues until culture day 7 (Fig. 3f), suggesting a possible return of the synthetic and proliferative M-SMC back to the contractile state by culture on rigid type IV collagen gels. In contrast, cells cultured on type IV collagen-coated surfaces showed no immunofluorescence (Fig. 3d), suggesting that the synthetic cells remain as they were seeded.

Contractility is one of the most basic functions of A-SMC. M-SMC which have lost their contractile ability might have regained contractility upon culturing on rigid type IV collagen gels (Fig. 4), suggesting that M-SMC re-differentiate on rigid type IV collagen gels.

What characteristics of the rigid type IV collagen gels are responsible for the specific effect on M-SMC? Gel rigidity or elasticity may be partially responsible. Our previous reports describe that the rigidity of type IV collagen gels depends greatly on protein concentration, NaCl concentration, incubation temperature, and length of incubation for gel formation (*27*). Thus, when the incubation time for gel formation is shortened, insufficiently rigid gels are formed. As shown in Fig. 1, a and d, cells cultured on fragile type IV collagen gels show a similar morphology to those cultured on dishes coated with type IV collagen solution, suggesting that the mechanical rigidity of the gel might be an important factor.

How far does the effect of the rigid type IV collagen gels extend to other differentiated cells? Rigid type IV collagen gels show similar effects on myofibroblast-like kidney mesangial cells and hepatic stellate cells (Hirose *et al.*, manuscript in preparation). That is, rigid type IV collagen gels repress growth and induce cellular morphologies that lead to the formation of a multicellular meshwork (data not shown). Mesangial cells, hepatic stellate cells, and A-SMC have several common characteristics such as contractility in response to external stimuli, production of collagenous proteins, tissue localization at a site underneath endothelial or epithelial cells, and phenotypic transformation into fibroblast-like cells (*41-46*).

M-SMC share many functional and morphological analogies with fibroblasts (*47*). M-SMC, like fibroblasts, contract collagen gels when cultured within reconstituted type I collagen gels. Cells that contribute to the pathogenesis of fibrosis or sclerosis in fibrotic or cirrhotic tissues by depositing fibrous materials are not well distinguished between fibroblasts and myofibroblasts. It is not easy to distinguish M-SMC or myofibroblasts from fibroblasts in *in vitro* culture or *in vivo*. However, as seen in the present report, human dermal fibroblasts show behaviors distinct from M-SMC in culture on rigid type IV collagen gels. The fibroblasts proliferate and show bipolar morphologies without cell-to-cell junctions at a later stage of culture on rigid type IV collagen gels (Figs. 5 and 6). Thus, by using rigid type IV collagen gels, M-SMC with myofibroblast-like phenotypes can be discerned from fibroblasts. The distinct response of M-SMC from fibroblasts would be due to an essential difference in cell receptor-mediated signal transduction for the rigid type IV collagen gel. The details remain to be elucidated.

In conclusion, the present study demonstrates some characteristic effects of rigid type IV collagen gels on the behavior of M-SMC: (i) multicellular formation with cell-to-cell connections, (ii) growth suppression, (iii) the expression of contractile marker proteins, and (iv) A-SMC contractility. The results suggest the entirely new possibility that rigid type IV collagen gels may help myofibroblast-like cells revert to quiescent and contractile A-SMC.

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REFERENCES

1. Thyberg, J., Hedin, U., Sjölund, M., Palmberg, L., and Bottger, B.A. (1990) Regulation of differentiated properties and proliferation of arterial smooth muscle cells. *Arteriosclerosis* **10**, 966-990

2. Ross, R. (1993) The pathogenesis of atherosclerosis: A perspective for the 1990s. *Nature* **362**, 801-809
3. Thyberg, J. (1996) Differentiated properties and proliferation of arterial smooth muscle cells in culture. *Int. Rev. Cytol.* **169**, 183-265
4. Chamley-Campbell, J. and Campbell, G.R. (1981) What controls smooth muscle phenotype? *Atherosclerosis* **40**, 347-357
5. Chamley-Campbell, J., Campbell, G.R., and Ross, R. (1979) The smooth muscle cell in culture. *Physiol. Rev.* **59**, 1-61
6. Chamley-Campbell, J.H., Campbell, G.R., and Ross, R. (1981) Phenotype-dependent response of cultured aortic smooth muscle to serum mitogens. *J. Cell Biol.* **89**, 379-383
7. Campbell, G.R. and Chamley-Campbell, J. (1981) The cellular pathobiology of atherosclerosis. *Pathology* **13**, 423-440
8. Rekhter, M.D. and Gordon, D. (1995) Active proliferation of different cell types, including lymphocytes, in human atherosclerotic plaques. *Am. J. Pathol.* **147**, 668-677
9. Lin, C.Q. and Bissell, M.J. (1993) Multi-faceted regulation of cell differentiation by extracellular matrix. *FASEB J.* **7**, 737-743
10. Jones, P.L., Schmidhauser, C., and Bissell, M.J. (1993) Regulation of gene expression and cell function by extracellular matrix. *Crit. Rev. Eukaryo. Gene Exp.* **3**, 137-154
11. Thoumine, O., Nerem, R.M., and Girard, P.R. (1995) Changes in organization and composition of the extracellular matrix underlying cultured endothelial cells exposed to laminar steady shear stress. *Lab. Invest.* **73**, 565-576
12. Berthiaume, F., Moghe, P.V., Toner, M., and Yarmush, M.L. (1996) Effect of extracellular matrix topology on cell structure, function and physiological responsiveness: Hepatocytes cultured in a sandwich configuration. *FASEB J.* **10**, 1471-1484
13. Thyberg, J., Palmberg, L., Nilsson, J., Ksiazek, T., and Sjölund, M. (1983) Phenotype modulation in primary cultures of arterial smooth muscle cells: On the role of platelet-derived growth factor. *Differentiation* **25**, 156-167
14. Hedin, U., Bottger, B.A., Forsberg, E., Johansson, S., and Thyberg, J. (1988) Diverse effects of fibronectin and laminin on phenotypic properties of cultured arterial smooth muscle cells. *J. Cell Biol.* **107**, 307-319
15. Thyberg, J. and Hultgårdh-Nilsson, A. (1994) Fibronectin and the basement membrane components laminin and collagen type IV influence the phenotypic properties of subcultured rat aortic smooth muscle cells differently. *Cell Tissue Res.* **276**, 263-271
16. Yamamoto, M., Yamamoto, K., and Noumura, T. (1993) Type I collagen promotes modulation of cultured rabbit arterial smooth muscle cells from a contractile to a synthetic phenotype. *Exp. Cell Res.* **204**, 121-129
17. Ehrlich, H.P., Griswold, T.R., and Rajaratnam, J.B. (1986) Studies on vascular smooth muscle cells and dermal fibroblasts in collagen matrices: Effects of heparin. *Exp. Cell Res.* **164**, 154-162
18. Yamamoto, M., Nakamura, H., Yamato, M., Aoyagi, M., and Yamamoto, K. (1996) Retardation of phenotypic transition of rabbit arterial smooth muscle cells in three-dimensional primary culture. *Exp. Cell Res.* **225**, 12-21
19. Bell, E., Ivarsson, B., and Merrill, C. (1979) Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential *in vitro*. *Proc. Natl. Acad. Sci. USA* **76**, 1274-1278
20. Yamato, M., Adachi, E., Yamamoto, K., and Hayashi, T. (1995) Condensation of collagen fibrils to the direct vicinity of fibroblasts as a cause of gel contraction. *J. Biochem.* **117**, 940-946
21. Nishiyama, T., Tsunenaga, M., Nakayama, Y., Adachi, E., and Hayashi, T. (1989) Growth rate of human fibroblasts is repressed by the culture within reconstituted collagen matrix but not by the culture on the matrix. *Matrix* **9**, 193-199
22. Hayashi, T., Yamato, M., Adachi, E., and Yamamoto, K. (1993) Unique features of type I collagen as a regulator for fibroblast functions: Iterative interactions between reconstituted type I collagen fibrils and fibroblasts in *New Functionally Materials, Volume B. Synthesis and Functional Control of Biofunctionality Materials* (Tsuruta, T., Doyama, M., Seno, M., and Imanishi, Y., eds.) pp. 239-246, Elsevier Science Pub. BV., Amsterdam
23. Koyama, H., Raines, E.W., Bornfeldt, K.E., Roberts, J.M., and Ross, R. (1996) Fibrillar collagen inhibits arterial smooth muscle proliferation through regulation of Cdk2 inhibitors. *Cell* **87**, 1069-1078
24. Adachi, E., Hopkinson, I., and Hayashi, T. (1997) Basement-membrane stromal relationships: Interactions between collagen fibrils and the lamina densa. *Int. Rev. Cytol.* **173**, 73-156
25. Kino, J., Adachi, E., Yoshida, T., Asamatsu, C., Yamamoto, K., and Hayashi, T. (1991) A novel chain of basement membrane-associated collagen as revealed by biochemical and immunohistochemical characterizations of the epitope recognized by a monoclonal antibody against human placenta basement membrane collagen. *Am. J. Pathol.* **138**, 911-920
26. Adachi, E., Takeda, Y., Nakazato, K., Muraoka, M., Iwata, M., Sasaki, T., Imamura, Y., Hopkinson, I., and Hayashi, T. (1997) Isolated collagen IV retains the potential to form an 18-nm sided polygonal meshwork of the lamina densa. *J. Electron Microsc.* **46**, 233-241
27. Nakazato, K., Muraoka, M., Adachi, E., and Hayashi, T. (1996) Gelation of the lens capsule type IV collagen solution at a neutral pH. *J. Biochem.* **120**, 889-894
28. Muraoka, M. and Hayashi, T. (1993) Three polypeptides with distinct biochemical properties are major α chain-size components of type IV collagen in bovine lens capsule. *J. Biochem.* **114**, 358-362
29. Yamato, M., Yamamoto, K., and Hayashi, T. (1992) Decrease in cellular potential of collagen gel contraction due to *in vitro* cellular aging: A new aging index of fibroblast with high sensitivity. *Connective Tissue* **24**, 157-162
30. Sakata, N., Kawamura, K., and Takebayashi, S. (1990) Effects of collagen matrix on proliferation and differentiation of vascular smooth muscle cells *in vitro*. *Exp. Mol. Pathol.* **52**, 179-191
31. Kocher, O. and Gabbiani, G. (1986) Expression of actin mRNA in rat aortic smooth muscle cells during development, experimental intimal thickening, and culture. *Differentiation* **32**, 245-251
32. Campbell, J.H., Kocher, O., Skalli, O., Gabbiani, G., and Campbell, G.R. (1989) Cytodifferentiation and expression of α-smooth muscle actin mRNA and protein during primary culture of aortic smooth muscle cells: Correlation with cell density and proliferative state. *Arteriosclerosis* **9**,633-643
33. Kuro-o, M., Nagai, R., Tsuchimochi, H., Katoh, H., Yazaki, Y., Ohkubo, A., and Takaku, F. (1989) Developmentally regulated expression of vascular smooth muscle myosin heavy chain isoforms. *J. Biol. Chem.* **264**, 18272-18275
34. Birukov, K.G., Frid, M.G., Rogers, J.D., Shirinsky, V.P., Koteliansky, V.E., Campbell, J.H., and Campbell, G.R. (1993) Synthesis and expression of smooth muscle phenotype markers in primary culture of rabbit aortic smooth muscle cells: Influence of seeding density and media and relation to cell contractility. *Exp. Cell Res.* **204**, 46-53
35. Shirinsky, V.P., Birukov, K.G., Koteliansky, V.E., Glukhova, M.A., Spanidis, E., Rogers, J.D., Campbell, J.H., and Campbell, G.R. (1991) Density-related expression of caldesmon and vinculin in cultured rabbit aortic smooth muscle cells. *Exp. Cell Res.* **194**, 186-189
36. Longtine, J.A., Pinkus, G.S., Fujiwara, K., and Corson, J.M. (1985) Immunohistochemical localization of smooth muscle myosin in normal human tissues. *J. Histochem. Cytochem.* **33**, 179-184
37. Hirschi, K.K. and D'Amore, P.A. (1996) Pericytes in the microvasculature. *Cardiovasc. Res.* **32**, 687-698
38. Yamato, M. and Hayashi, T. (1998) Topological distribution of collagen binding sites on fibroblasts cultured within collagen gels in *Extracellular Matrix-Cell Interaction: Molecules to Diseases* (Ninomiya, Y., Olsen, B.R., and Ooyama, T., eds.) pp. 123-140, Japan Sci. Soc. Press, Tokyo
39. Birukov, K.G., Stepanova, O.V., Nanaev, A.K., and Shirinsky, V.P. (1991) Expression of calponin in rabbit and human aortic smooth muscle cells. *Cell Tissue Res.* **266**, 579-584
40. Woodcock-Mitchell, J., Mitchell, J.J., Low, R.B., Kieny, M., Sengel, P., Rubbia, L., Skalli, O., Jackson, B., and Gabbiani, G. (1988) α-Smooth muscle actin is transiently expressed in embry-

onic rat cardiac and skeletal muscles. *Differentiation* **39**, 161-166
41. Singhal, P.C., Scharschmidt, L.A., Gibbons, N., and Hays, R.M. (1986) Contraction and relaxation of cultured mesangial cells on a silicone rubber surface. *Kidney Int.* **30**, 862-873
42. Sakamoto, M., Ueno, T., Kin, M., Ohira, H., Torimura, T., Inuzuka, S., Sata, M., and Tanikawa, K. (1993) Ito cell contraction in response to endothelin-1 and substance P. *Hepatology* **18**, 978-983
43. Nerlich, A. and Scheinman, E. (1991) Immunohistochemical localization of extracellular matrix components in human diabetic glomerular lesions. *Am. J. Pathol.* **139**, 889-899
44. Geerts, A., Vrijsen, R., Rauterberg, J., Burt, A., Schellinck, P., and Wisse, E. (1989) *In vitro* transition of fat-storing cells to myofibroblast-like cells parallels marked increase of collagen synthesis and secretion. *J. Hepatol.* **9**, 59-68
45. Milani, S., Herbst, H., Schuppan, D., Hahn, E., and Stein, H. (1989) *In situ* hybridization for procollagen type I, III and IV mRNA in normal and fibrotic rat liver: Evidence for predominant expression in nonparenchymal cells. *Hepatology* **10**, 84-92
46. Wake, K. (1980) Perisinusoidal stellate cells (fat-storing cells, interstitial cells, lipocytes), their related structure in and around the liver sinusoids, and vitamin A-storing cells in extrahepatic organs. *Int. Rev. Cytol.* **66**, 303-353
47. Desmouliére, A. and Gabbiani, G. (1995) Smooth muscle cell and fibroblast biological and functional features: Similarities and differences in *The Vascular Smooth Muscle Cell* (Schwartz, S.M. and Mecham, R.P., eds.) pp. 329-359, Academic Press, San Diego