*Transactions of the Materials Research Society of Japan* 25 [4] 907-910 (2000)

# Implications for Biological Functions of Type IV Collagen Gels

Motohiro Hirose, Kazunori Mizuno, Hiroaki Kosugi, Kiwamu Yoshikawa,
Hiroko Yamano, Takanori Kihara, and Toshihiko Hayashi

Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo,

Komaba 3-8-1, Meguro-ku, 153-8902 Tokyo, Japan

Fax: 81-3-5454-6998, e-mail : cthayas@mail.ecc.u-tokyo.ac.jp

It was found that human arterial smooth muscle cells (A-SMC) that had acquired a proliferative phenotype through cell passages in sera were most strongly affected by the culture substrate. The cells are referred to as myofibroblast-like cells (M-SMC). When the M-SMC were cultured on the type I collagen gel, the initiation of cell growth was retarded. However, the cells eventually acquired proliferative potential within a few days in culture. The primary A-SMC, as previously reported, may maintain non-proliferative phenotype in culture on the type I collagen gel without serum. However, the growth repressive effect of the type IV collagen gel was found in the culture with serum for a prolonged time up to weeks or more. Furthermore, the cells with proliferative phenotype went back to a quiescent and contractile phenotype by the culture on the type IV collagen gel. On the contrary, the cell proliferated most rapidly with a fibroblastic phenotype by culture on the dish coated with type IV collagen solution. The results indicated that the smooth muscle phenotypes are influenced by physical states of the type IV collagen, suggesting that three-dimensional structure of type IV collagen at the interface with the cells may be critical.

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

## 1. Introduction

The functions of aortic smooth muscle cells (A-SMC) are affected and thus regulated in cell culture by extracellular matrix (ECM) used as consolidated culture substrates (1, 2). In the blood vessel wall, endothelial cells, A-SMC, and fibroblasts are aligned along with type IV collagen, type V collagen, and type I collagen.

We have hypothesized that the specific interrelation between cell types and consolidated environments consisting of collagen aggregates has some biological meanings. The hypothesis was tested by asking whether cell specific phenotype can be affected in different ways by the reconstituted collagen aggregates used as cell culture substrates. The regulation of smooth muscle phenotypes is considered to be crucial in the pathogenesis of fibrosis of various organs. A similar change to a fibroblastic phenotype occurs in A-SMC during prolonged periods of primary culture *in vitro* and repeated passages in serum culture (1, 3-5). Recently it was reported that fibrillar type I collagen (6) or the gel forms of type I and type III collagen (7) retard A-SMC proliferation in primary cultures without serum. We found that the type IV collagen solution gels under selected conditions of NaCl concentration and temperature upon formation of the polygonal meshwork (8). We here report the effects of the type IV collagen gel 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.

## 2. Experimental Procedures

*2-1. Preparation of lens capsule type IV collagen :* 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 (9). Briefly, lens capsules were homogenized in 0.5 M acetic acid containing protease inhibitors. After centrifugation at 800×g for 15 min, the supernatant was collected and dialyzed against 1 mM HCl and stored at 4°C before use. Protein concentrations were determined by the weight of the lyophilized material.

*2-2. Cell culture:* Human A-SMC at passage 3 were purchased from Clonetics Corp. (San Diego, CA, USA). The cells were cultured in modified MCDB131 medium (Clonetics Corp.) supplemented with 10% FBS

(Growth medium) at 37°C and 5% $CO_2$. M-SMC obtained from A-SMC by serial passages up on 100 mm dishes were cultured on different substrates.

*2-3. 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 (10). Successive culture of the cells on type I collagen gel were performed as previously described (10). 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 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 $7.2\times10^3$ cells was plated on each gel and the cells were grown at 37°C. The preparation of type IV collagen gels has been described previously (8). 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. After replacing the buffer with Growth medium, 1 ml of cell suspension containing $7.2\times10^3$ cells was placed on each gel and the cells were cultured at 37°C. 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. The neutralized solution was added to 24-well dishes (500 μl/ well) and then dried at 25°C. 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 2h. The wells were then washed with Growth medium. Cell morphology was observed with a phase-contrast microscope (Model DMIRB, Leica Co., Tokyo) at 100× magnification.

*2-4. Cell proliferation assay* : Cells cultured on type I collagen and type IV collagen gels were washed with PBS (-) and treated with 500 μl of 0.2% bacterial collagenase (Wako Pure Chemicals Inc., Tokyo) containing 1 mM $CaCl_2$ in PBS (-) at 37°C for 2 h to suspend the cells. Cells cultured on plastic 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 were counted in triplicate wells on day 2, 5, and 10 with a Coulter counter (Z-1, Coulter Co., Tokyo).

*2-5. Immunocytochemistry* : M-SMC cultured on dishes coated with type IV collagen solution, type I collagen gel, or 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 Co., 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 Co., Tokyo) in fluorescence mode at 400× magnification. Non-immune mouse IgG (ICN Pharmaceuticals, Inc., USA) was used as a control in place of specific antibodies against SMH.

## 3. Results

*3-1. 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. 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 five different substrates as follows: 1) dishes coated with type I collagen solution, 2) dishes coated with type IV collagen solution (Fig. 1a), 3) type I collagen gel (Fig. 1b), 4) dishes coated with aggregated type IV collagen (non-gel form) (Fig. 1c), 5) type IV collagen gel with sufficient rigidity (Fig. 1e, f) and observed them at culture day 3. Once the cells started to elongate on type IV collagen gels, 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, resulting in a mesh-like multicellular organization as shown in Fig. 1e. Type IV collagen gel s was sometime fragile due to shortage of incubation time. The cell shape on fragile type IV collagen gel was similar to that on type IV collagen-coated dish; bipolar cell shape (Fig. 1d).

*3-2. Proliferation of M-SMC in culture* : 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 type IV collagen gels (Fig. 2). 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.

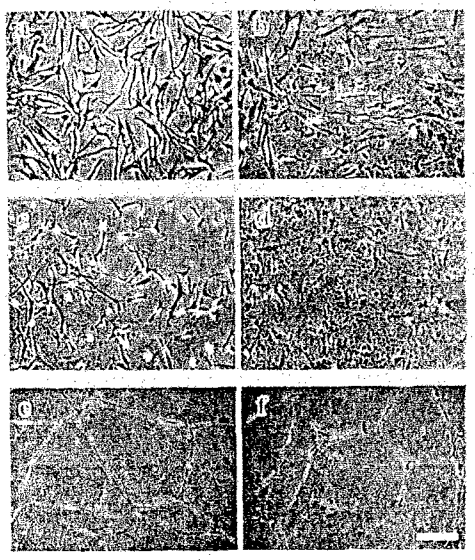

Fig. 1. Morphology of M-SMC cultured on different substrates: M-SMC obtained from A-SMC by repeated passages up to 9 PDL were cultured on type IV collagen-coated dishes (a), type I collagen gels (b), aggregated type IV collagen-coated dishes (c), type IV collagen gels with insufficient rigidity (d), or type IV collagen gels with sufficient rigidity (e, f). At culture day 3 (a-e) or day 14 (f), the morphology of the M-SMC was observed by phase-contrast microscopy and photomicrographed. Bar; 100 μm.

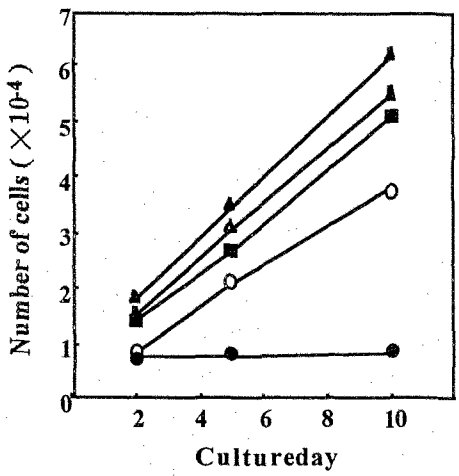


Fig. 2. Growth curves of M-SMC cultured on different substrates: M-SMC obtained from A-SMC through repeated passages up to 9 PDL were cultured on plastic dishes (■), type I collagen-coated dishes (△), type IV collagen-coated dishes (▲), type I collagen gels (○), or rigid type IV collagen gels (●) in Growth medium for 10 days. The cells were initially plated at a cell density of $7.2\times10^3$/ 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.

*3-3. Expression of marker proteins for contractile stage A-SMC in culture :* Smooth muscle myosin heavy chains (SMH) is generally assumed to be the most specific and reliable marker for the contractile stage of A-SMC (11). 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 the rigid type IV collagen gels (Fig. 3f).

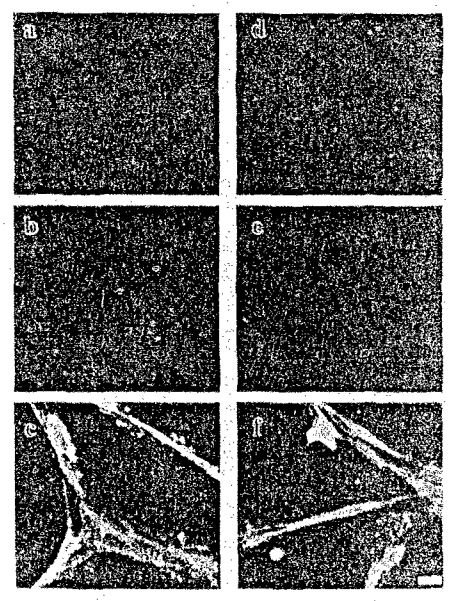

Fig. 3. Expression of SMH in cultured M-SMC: M-SMC obtained from A-SMC through repeated passages up to 13 PDL were cultured on type IV collagen-coated dishes (a, d), type I collagen gels (b, e), or rigid type IV collagen gels (c, f) for 3 days (a-c) or 7 days (d-f). The cells were fixed and permeabilized 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.

## 4. Discussion

The morphological characteristics of M-SMC on the type IV collagen gels can be summarized as follows: 1) highly elongated shape, 2) side-by-side association of the elongated cells, and 3) cell-to-cell contacts at the tips of adjacent cells, presumably with the formation of cell-to-cell junctions (Fig. 1).

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 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). 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.

SMH seems to be specific for A-SMC in the contractile state among other biochemical markers (11, 12). Immunocytochemical analyses with anti-SMH antibody revealed that 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 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.

What characteristics of the 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 on protein concentration, NaCl concentration, incubation temperature, and length of incubation for gel formation (8). Thus, when the incubation time for gel formation is shortened, insufficiently rigid gels are formed. The cells cultured on fragile type IV collagen gels show a similar morphology to those cultured on type IV collagen-coated dishes (Fig. 1d), suggesting that mechanical rigidity of gels might be an important factor.

In conclusion, the present study demonstrates some characteristic effects of type IV collagen gels on the behavior of M-SMC: 1) multicellular formation with cell-to-cell connections, 2) growth suppression, and 3) the expression of contractile marker proteins. The results suggest the entirely new possibility that type IV collagen gels may help myofibroblast-like cells revert to quiescent and contractile A-SMC.

Acknowledgment

The present study was supported in part by a Scientific Research Grant from the Ministry of Education, Culture, Sports and Sciences of Japan: Grant-in-Aid for Scientific Research on Priority Areas (09229219, 10123207 Functionally Graded Materials, 09217210 Supramolecular Structure), a Grant-in-Aid for Developmental Scientific Research (07558249), by The Japan Society for the Promotion of Science, "Research for the Future" Program (JSPS-RFTF96I00201), and by the Program for the Promotion of Fundamental Studies in Health Sciences from The Organization for Pharmaceutical Safety and Research (OPSR).

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(Received December 16, 1999; Accepted September 30, 2000)