Biological functions of type Ⅳ collagen

(Ⅳ型コラーゲンの生物学的機能)

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Biological functions of type IV collagen

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by

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Contents

Abbreviations

A-EC:	aortic endothelial cell
A-SMC:	aortic smooth muscle cell
α-SMA:	α-smooth muscle actin
BSA:	bovine serum albumin
DMEM:	Dulbecco's modified Eagle medium
ECM:	extracellular matrix
EDTA:	ethylenediaminetetraacetic acid
FBS:	fetal bovine serum
FITC:	fluorescein isothiocyanate
G-MC:	glomerular mesangial cell
HF:	human dermal fibroblast
HSC:	hepatic stellate cell
MDCK:	Mardin Darbyn caine kidney cell
M-HSC:	myofibroblast-like hepatic stellate cell
M-MC:	myofibroblast-like mesangial cell
M-SMC:	myofibroblast-like aortic smooth muscle cell
PAGE:	polyacrylamide slub gel electrophoresis
PBS (-):	Ca^{2+}-and Mg^{2+}-free Dulbecco's phosphate-buffered saline
PDL:	population doubling level
RD:	rhabdomyosarcoma cell
SDS:	sodium dodesyl sulfate
SMH:	smooth muscle myosin heavy chain

Preface

It has been shown that cell fundamental functions including growth, maintenance of differentiation, gene expression, cell migration as well as cell attachment are affected by extracellular matrix (ECM). The direct evidence was obtained through the studies on the effects of cell culture substrate on the cultured cells (Jones et al., 1993; Lin et al., 1993; Thoumine et al., 1995; Berthiaume et al., 1996). Collagenous proteins represent most abundant components of ECM particularly large animals, suggesting that the collagenous proteins, in particular supramolecular aggregates may afford body or tissue skeletal structures. They may also be consolidated regulators of cellular functions.

Isolated type I collagen, type IV collagen and type V collagen formed characteristic supramolecular assemblies in the electron microscopic observations under selected conditions (Adachi et al., 1994; 1997a; 1997b; Hirose et al., 1997). Type I collagen forms fibrils of varying diameters with branching; Type IV collagen forms polygonal meshwork with pores of 18 nm in diameter; and Type V collagen forms thin-width tape-like fibrils without branching. Apparent similarities of the reconstituted aggregate structures to *in vivo* supramolecular structures as well as immunohistochemical localization of different types of collagen have allowed me to hypothesize that the major three collagen types may comprise the scaffoldings of various tissues in a graded manner as shown in Fig. 1 (Adachi et al., 1997a). The order of cell types lining from endothelial cells, smooth muscle-related cells and fibroblasts may correspond to the graded collagen aggregates from type IV collagen, type V collagen, and type I collagen. The hypothesis that the graded structure of collagen aggregates has a biological function to maintain the arrangement of differentiated cell types could be tested by whether smooth muscle-related cells, endothelial cells and epithelial cells that are presumed in contact with type IV collagen have preferences for type IV collagen in culture system.

Another way of looking at possible characteristics of collagenous proteins as consolidated regulators would be the physical properties of collagen aggregates; elasticity, plasticity, or permeability of small molecules. My particular interest in this relation is whether the collagen aggregates are in a gel form or not, since the primary solvent in the living organisms is water that interacts with collagenous proteins in a unique way.

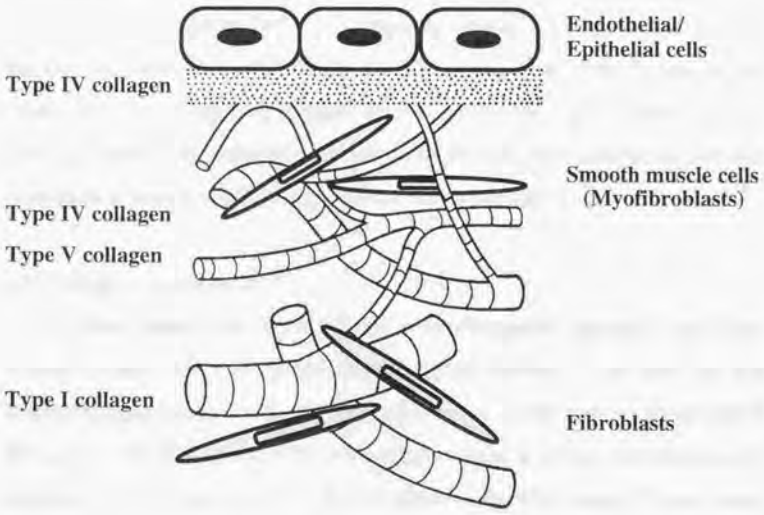


Fig. 1. A graded structure model of the collagen aggregates with three differentiated cells

General Introduction

1.1 Extracellular matrix

Extracellular matrix (ECM) is the assembly of organic components and defined as the extracellular environments which facilitate the formation and maintenance of multicellular system as a functional cell society. The extracellular matrix thus in large vertebrates such as humans serves as solid-state regulators for cellular function as well as scaffolding of the tissue architecture or the infrastructures of large animals.

ECM components can be classified into following four groups from the biochemical characteristics (Kielty et al. 1993); (1) collagen superfamily, (2) glycoproteins, such as laminin superfamily, fibronectin, fibrillin, thrombospondin, tenascin etc, (3) proteoglycan superfamily, such as aggrecan, perlecan, decorin, syndecan etc., and (4) others, such as elastin and hyaluronan. Collagen superfamily is the most abundant in the whole body and considered to form main scaffolding of various tissues and organs.

1.2 Collagen superfamily

The main characteristics of the collagen is the collagenous left-handed triple helix formed through Gly-X-Y triplets with abundant proline residues on X positions and rich 4-hydroxyproline residues on Y positions. Conformation of each chain is a polyproline-II like right-handed helix. Some of the triple-helical domains including that of the type IV collagen have interruptions in the Gly-X-Y triplets in that Gly residues do not occupy every third position. There are at least 30 different chains and more than 19 types in the collagen family. Collagen superfamily is subclassified into subgroups from the molecular and/or supramolecular structure (Jacenko et al., 1991) ; (1) fibrillar collagens (type I, II, III, V and XI) that have the property to form fibrils in the interstitial ECM, (2) basement membrane collagens, type IV collagens comprises a family of at least genetically distinct six α chains, (3) FACIT (fibril associated collagen with interrupted triple helices) collagen

(type IX, XII, XIV and XVI) and so on. The most abundant collagen in vertebrates is type I collagen which exist most of all the tissues except for cartilage. The cartilage mainly contains type II and type XI collagen. From the histochemical or biochemical studies, type IV collagen mainly exists in basement membrane and also exists around stromal cells including aortic smooth muscle cells (A-SMC) in artery, hepatic stellate cells (HSC) in liver and glomerular mesangial cells (G-MC) in kidney. In all cases, collagenous proteins forms fine supramolecular aggregates *in vivo*. The supramolecular aggregates comprising collagens, or collagen and other ECM components would be the characteristic structural elements for the biological functions.

2.1 Type IV collagen molecules and type IV collagen gel

Type IV collagen is one of the major components of basement membrane. Type IV collagen also exists around the stromal cells such as A-SMC (Kino et al., 1991), HSC (Yoshida et al., 1997) and G-MC (Nerlich et al., 1991). Type IV collagen was discovered as the collagenous polypeptides extracted from glomerulus, Descemet's membrane and lens capsule with pepsin digestion (Kefalides, 1968). The major type IV collagen molecule has a chain composition of $[\alpha 1(IV)]_2\alpha 2(IV)$. Type IV collagen molecules are degraded with pepsin rather readily in comparison with the fibrillar collagens. It is generally considered that the degradation may be related to destabilized helices due to interrupted triplet sequences of Gly-X-Y, where X and Y indicate any amino acid residues. The type IV collagen molecules with any chain compositions comprise three distinct domains; 1) non-collagenous region (NC1), 2) major triple helical region with interrupted Gly-X-Y and 3) 7S region (Timpl et al., 1981).

Type I collagen, type II collagen, and type III collagen also retain propeptides on both sides of the triple helices when they are secreted from cells. As the propeptides are processed, the molecules are deposited in the solid phase. On the other hand, type IV collagen retains extension on both ends of the molecule after the secretion, NC1 region on

the carboxyl end and 7S region on the amino terminal end. From the studies of EHS tumor type IV collagen, the extracted type IV collagen polypeptides from deposits consisted of 185 K of α1(IV) and 175 K of α2(IV) (Yurchenco et al., 1984). On the other hand, it has been reported that type IV collagen polypeptides extracted from lens capsules contained short form, 160 K α1(IV) which lacked 7S region (Muraoka et al., 1993; Iwata et al., 1995; 1996). Sasaki et al. showed that short chain existed not only in lens capsule but also in human placenta, bovine kidney and cultured cell layer (Sasaki et al., manuscript in preparation). These results indicated that type IV collagen deposited in the tissues examined except EHS tumor or cell layers at a later stage of cell culture contained the short form of α1(IV) and α2(IV) chains. Six subtypes of type IV collagen chains (α1-α6) have been discovered until now (Ohashi et al., 1995). Among these, more than 80 % of type IV collagen detected in matured tissues is considered to be $[\alpha1(IV)]_2$ α2(IV). In the case of lens capsule tissues, 94 % of the extracted NC1 domain consisted of α1(IV) and α2(IV) (Kahsai et al., 1997; Langeveld et al., 1988).

Lens capsule type IV collagen aggregated into a gel in 2 M guanidine-HCl with 50 mM dithiothreitol (Muraoka et al, 1996), where most of the biological macromolecules are to be dissociated. The gel formation of bovine lens capsule type IV collagen also took place at physiological pH and NaCl concentration (Nakazato et al., 1996). Nakazato et al. evaluated mechanical rigidity or fragility of the type IV collagen gel by measuring the relative reduction in volume after centrifugation. Rigidity of the type IV collagen gel depended on the gelation conditions such as NaCl concentration, temperature, protein concentration and incubation time (Fig. 2; p. 12, 13). The results they obtained indicated that 1) 150 mM NaCl was optimal, 2) Incubation temperature was low up to 4°C and 3) Protein concentration more than 1 mg /ml was necessary for apparent rigidity of the gel. Moreover, the longer the incubation time was, the more rigid was the gel formed.

Elevated temperature up to 37°C of the type IV collagen gel formed at 4°C throughout gelation did not cause the change in the rigidity of the gel, indicating that the type IV collagen gel could be used as cell culture substrate under the conventional culture condition of 5% CO_2-95% air at 37°C. In this work, in order to obtain the rigid type IV collagen gel to accommodate cells on top of the gel, type IV collagen solution with sufficiently high concentration (2 mg /ml) was incubated at 4°C for a long time (more than 5 days). Nakazato et al. observed the supramolecular structure of type IV collagen gel under an electron microscope (Fig. 3; p. 14). Branching and anastomosing of filaments were seen in the gels formed at 4°C and also at 28°C. The type IV collagen meshwork has a pore size of about 18 nm (Adachi et al., 1997), resembling in dimension the basal lamina skeletal meshwork of mouse pancreas *in vivo*. A fine meshwork of type IV collagen aggregate seems to originate from branching at the molecular level, which could be the triple-helical interruption sites. Under the conditions, the type I collagen comprising the triple-helical domain alone remains dispersed in solution. The unique intermolecular interaction of the type IV collagen resulting in gel formation in guanidine-HCl+DTT at cold is ascribed to the NC1 domain, since NC1 domain-free type IV collagen obtained by pepsin or chymotrypsin treatment did not form gel. In this thesis, I used type IV collagen gel with sufficient rigidity and with insufficient rigidity (in Chapter III) reconstituted from bovine lens capsule type IV collagen under 20 mM phosphate containing 150 mM NaCl as cell culture substrate.

2.2 Biological functions of type IV collagen implicated from the previous studies

Type IV collagen provides a biomechanically stable scaffold, into which the other constituents of basement membranes are incorporated. Several studies indicated that some kinds of cells including keratinocytes (Murray et al., 1979), hepatocytes (Rubin et al., 1981), endothelial cells (Palotie et al., 1983; Tsilibary et al., 1990) and tumor cells

(Aumailley et al., 1986; Dennis et al., 1982) attached and responded to type IV collagen substrates. Recently, the two integrin receptors $\alpha 2\beta 1$ and $\alpha 1\beta 1$ have been reported to recognize the human $[\alpha 1(IV)]_2$ $\alpha 2(IV)]$ molecule (Eble et al., 1996; Vandenberg et al., 1991). However, less is known about other potential biological properties. No attempt is found to examine the type IV collagen gel on cell behaviors. One of the reasons is that commercially available type IV collagen is a pepsin treated one that can not form a gel.

2.3 Biological functions of type I collagen gel

Since the ECM comprises solid-state or assembled supramolecular structures and shows a potent activity in modifying the cellular functions, including proliferation and differentiation of the cells, the ECM could be defined as a solid-phase cytokine. It is interesting to know the causes of the effect of consolidated ECM on the cell behavior by locally elevated concentration or by organized arrangement of the macromolecules. In case of the type I collagen as substrate, the fibrillar structure showed some distinct effects that can not be acquired by the monomeric molecules. Particularly type I collagen gel showed marked effects on fibroblast behaviors.

Fibroblasts cultured in type I collagen gel showed 1) repressed growth (Schor, 1980; Weinberg et al., 1985; Nishiyama et al., 1989; Hayashi et al., 1991), 2) repressed production of collagenous proteins (Hayashi et al, 1991) and 3) repressed response to various growth factors or cytokines (Colige et al., 1988; Nishiyama et al., 1991; Hayashi et al., 1993). Elongated morphology was taken by the fibroblasts cultured within type I collagen gel (Tomasek et al., 1984; Nishiyama et al., 1993; Yamato et al., 1995; Mercier et al., 1996), although the change in cell shape was retarded. The dramatic effect of type I collagen gel has been extended to other cells such as vascular endothelial cells. Vascular endothelial cells organize rapidly into a network of capillary-like tubes when they were cultured within type I collagen gel (Montesano et al., 1983).

3. Cell culture substrates comprising graded structure of collagen supramolecular aggregates

The effects of ECM on the cellular functions have been investigated by using fibronectin or laminin including their functional domains as cell culture substrates, demonstrating that cell attachment to these ECM components via integrins involved in the regulation of cellular functions such as morphology, growth, differentiation and apoptosis. In contrast, the significant effects of collagen on the cellular functions have not been reported as found for fibronectin and laminin. Thus, the elucidation of biological functions of collagen should require an essentially distinct approach from conventional examinations dealing with molecules and peptides. Based on the anatomical and histological examinations of collagen aggregates, Adachi et al. have proposed a model of tissue graded structure in general in terms of the specific structure of different types of collagen aggregates (Adachi et al., 1997). The ultrastructure of various tissues matched well the reconstituted aggregate structures from isolated type I collagen, type V collagen and type IV collagen. Mesenchymal cells with different functions are also located in a graded way in accord with the graded distribution of collagen supramolecular aggregates. In the case of the blood vessel wall, the order of cell types lining from endothelial cells, smooth muscle cells and fibroblasts may correspond to the graded collagen aggregates from type IV collagen, type V collagen and type I collagen (Fig. 1; p. 2). *In vivo* tissues, assemblies of collagenous proteins, presumably a gel form, were thought to regulate and influence appropriate cell functions by continuous signals. In this work, I assumed the supramolecular structure comprising collagen aggregates might show biological functions of collagen. The cell proliferation, shape and responses to growth factors of the cultured fibroblasts were greatly affected by type I collagen gel as described in this thesis (General Introduction 2-3). Fibroblasts cultured in type I collagen gel expressed the phenotype as found in normal tissue. The findings have tempted me to examine whether this can be extended further to combination type IV collagen gel and smooth muscle-related cells.

4. Stromal cells

4-1 Hepatic stellate cells

In normal liver, hepatic stellate cells (HSC) are located underneath the endothelial cells in the perisinusoidal space of Disse. HSC have several specific functions; 1) Uptake, storage and release of retinoids, 2) synthesis and secretion of ECM proteins including type I, III, IV, V and VI collagen, fibronectin, laminin, tenascin and proteoglycans, 3) production of matrix metalloproteinases as well as tissue inhibitors of metalloproteinases, 4) synthesis and secretion of cytokines and growth factors, 5) contraction of the sinusoidal lumen in response to endothelin-1, angiotensin-II or prostaglandins (Wake, 1980; Milani et al., 1989; Blomhoff et al., 1991; Kawada et al, 1992; Sakamoto et al., 1993; Geerts et al., 1994). HSC extremely spread three dimensionally and connect with perisinusoidal endothelial cells as well as hepatocytes (Wake, 1988; 1995). ECM components regulate HSC cell shape, growth, differentiation and gene expression (Davis et al., 1987a; Friedman et al., 1985; 1989; Senoo et al., 1994). Studies in liver suggest that HSC under appropriate stimulation, modulate from quiescent (contractile) cells to activated (synthetic), acquiring morphological changes that mark them as myofibroblasts (Kent et al., 1976; Minato et al., 1983; Geerts et al., 1989; Maher et al., 1989; Schmitte-Gräff et al., 1991). The cells actively proliferate and secrete excessive fibrillar collagens. The mechanism of HSC activation represents a major issue with respect to the pathogenesis and to potential points of therapeutic intervention in fibrotic diseases. Studies with cell culture system may provide a potent tool to approach these problems experimentally. However, there is no source of the HSC maintaining contractile state for a sufficiently long time in culture. Establishment of the extracellular environments for keeping the HSC quiescent and furthermore for restoring the myofibroblastic state to the contractile state has not been possible. In Chapter I, I examined the effects of the type IV collagen gel on cell shape and proliferation of HSC in primary culture. Next in Chapter II, I investigated those in the case of myofibroblast-like cells.

4-2 Aortic smooth muscle cells

The aortic smooth muscle cell (A-SMC) is the sole cell type normally found in the media of animal arteries. In the adult, it is a terminally differentiated cell that expresses cytoskeletal marker proteins like α-smooth muscle actin and smooth muscle myosin heavy chains, and contracts in response to chemical and mechanical stimuli (Kocher et al., 1986; Kuro-o et al., 1989; Glukhova et al., 1990; Kanda et al., 1994; Levin, 1995). In atherosclerotic lesions, the cells revert to a proliferative and secretory state (Chamley-Campbell et al., 1981a). A similar transition from a contractile to a synthetic phenotype occurs *in vitro* culture. Thus, an *in vitro* culture system has been used to study the regulation of differentiated properties and proliferation of the cells. During the first few days, the cells morphologically change to fibroblast-like cells with a loss of myofilaments and formation of a large endoplasmic reticulum and a prominent Golgi apparatus. At the same time, they start to produce mitogens and secrete ECM components such as collagen, elastin and proteoglycans (Chamley-Campbell et al., 1981b; Thyberg et al., 1983; 1985; Palmberg et al., 1985). Based on the similar alteration *in vivo* and *in vitro* to the case of HSC, A-SMC and HSC are homologous cells in that they would be responsible for the tissue scleroses such as fibrosis or cirrhosis in liver and atherosclerosis in artery. Moreover in kidney, there exists glomerular mesangial cells (G-MC) with common features in A-SMC and HSC (Raines et al., 1991; Rojkind, 1991; Kopp et al., 1992). The mechanism underlying the change in morphology and function of A-SMC, HSC and G-MC from contractile to synthetic has been rather well studied by utilizing the culture system. For example, fibronectin substrate facilitated the change to synthetic phenotype (Hedin et al., 1988; Thyberg et al., 1994). However, the mechanism or even possibility of the change from synthetic to contractile is only poorly understood. It might sometimes thought that the change to fibroblastic state is irreversible. The proliferative and secretory activities of these cells are influenced by growth factors and ECM (Friedman et al., 1989; Thyberg et al., 1990; Saito et al., 1993; Yamamoto et al., 1993; 1996). Much work

remains for establishment of effective culture system. In Chapter III, the influence of the type IV collagen gel on the differentiation of myofibroblast-like SMC was studied.

Aims

In this thesis, I intended to evaluate the biological functions of the type IV collagen as an effective factor in regulating cellular functions including cell shape or cell growth which smooth muscle-related cells expressed when they were cultured on the substrates of the type IV collagen.

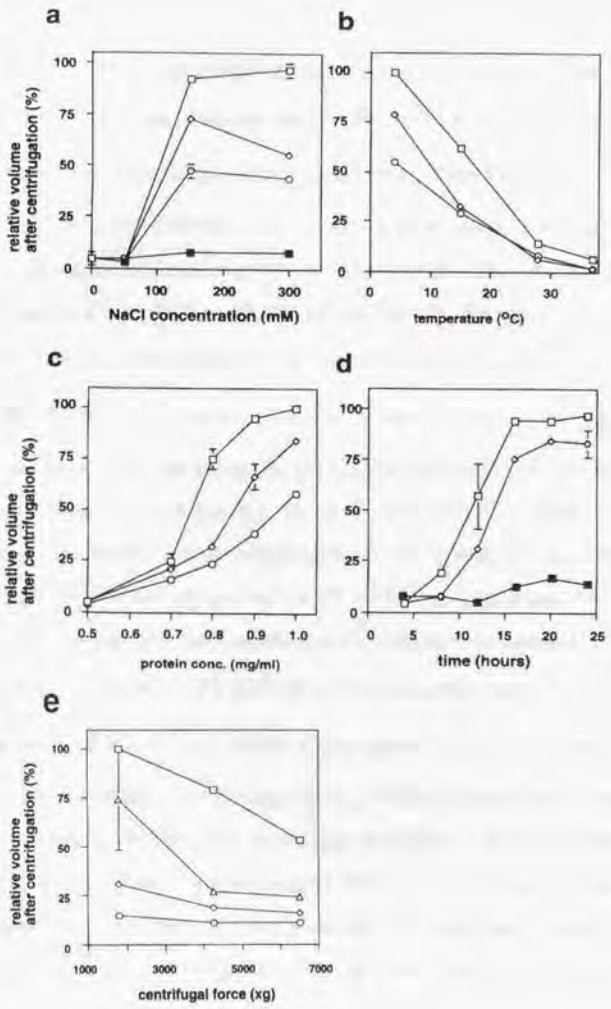


Fig. 2. Effects of NaCl concentration, temperature, protein concentration and incubation time on the rigidity of type IV collagen gel. This figures were cited from the article of Nakazato, K. et al. (1996), pp. 891-892. Figure legend is described in the next page.

Legend to Fig. 2.

The type IV collagen solution was incubated under the conditions indicated below. Then, the relative volume after centrifugation (□, ■, 1800×g; ◇, 4250×g; ○, 6500×g for 10 min) was measured. (a) Effect of ionic strength. Type IV collagen solution in acidic pH was mixed with 4 M NaCl and 200 mM phosphate, pH 7.3, in a glass tube to give a final phosphate concentration of 20 mM, a final protein concentration of 0.84 mg /ml and a final NaCl concentration of 0, 50, 150, or 300 mM. The relative volume of the gels was measured after centrifugation of the gels incubated for 24h at 4°C (□, ◇, ○) or 28°C (■). (b) Effect of temperature. Solutions of type IV collagen were made to give final concentrations of 20 mM phosphate, pH 7.3, 150 mM NaCl and 0.99 mg of the protein /ml, followed by incubation at 4, 15, 28, or 36°C for 24h. (c) Effect of protein concentration. The type IV collagen solutions (0.99-0.49 mg /ml) were incubated in 20 mM phosphate, pH 7.3, and 150 mM NaCl at 4°C for 24h. (d) Time course of the change in gel property. Solutions of lens capsule type IV collagen were incubated in 20 mM phosphate, pH 7.3, 150 mM NaCl, 0.99 mg of the protein /ml at 4°C (□, ◇), or 28°C (■) for 4-24h. (e) Effect of temperature on the gel rigidity. The type IV collagen solution was mixed at 4°C with 200 mM phosphate, pH 7.3, 4 M NaCl to give final concentration of 20 mM phosphate, 150 mM NaCl and 0.99 mg /ml protein, followed by incubation at 4°C or 28°C for 24h. Then, the temperature of incubation was changed and incubation was continued for another 24h. The relative volumes after centrifugation were measured (□, 4→4°C; △, 4→28°C; ◇, 28→4°C; ○, 28→28°C). Each point is the mean of two independent experiments.

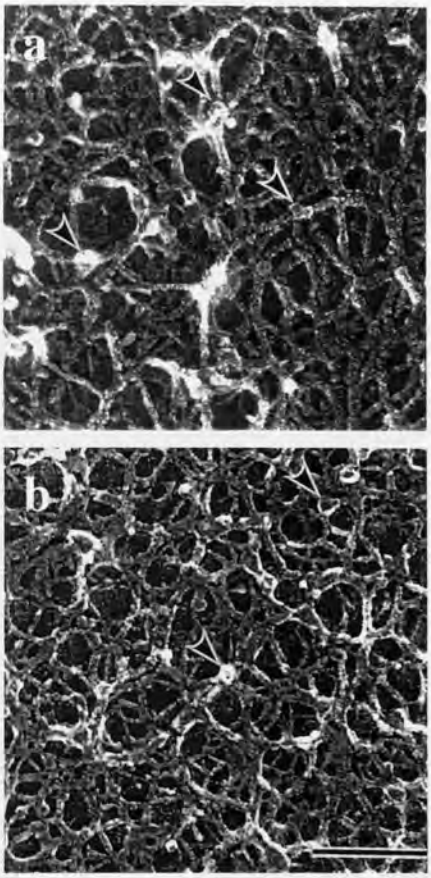


Fig. 3. Quick-freeze deep-etch replica image of type IV collagen gels. Type IV collagen solution was incubated in 20 mM phosphate buffer, pH 7.3, containing 150mM NaCl at 4°C (a) or 28°C (b) for more than 24 h. The gels formed were quick-frozen, deep-etched and rotary shadowed with Pt/C. The replicas show meshworks with extensive branching and anastomosing as shown in a and b. Globular structures (arrowheads) are seen on the filaments in a and b. The magnification is ×210,000. Bar; 100 nm. This figures were cited from the article of Nakazato, K. et al. (1996), p. 892.

Experimental Procedures

Cultured cells

Human aortic smooth muscle cells (A-SMC) at passage 3, human glomerular mesangial cells (G-MC) at passage 3 and human aortic endothelial cells (A-EC) at passage 3 were purchased from Clonetics Corp., San Diego, CA, USA. Myofibroblast-like smooth muscle cells (M-SMC) and myofibroblast-like mesangial cells (M-MC) were obtained from A-SMC by 3-5 times passages up to 9-13 PDL on the 100 mm dishes (Falcon, No.3003). Human rhabdomyosarcoma (RD) and Mardin Darbyn caine kidney cells (MDCK) were purchased from Health Science Research Resources Bank, Osaka. Normal human dermal fibroblasts (HF-18) were kindly gifted by Dr. Toshio Nishiyama of Shiseido Research Center, Yokohama. Rat hepatic stellate cells (HSC) were isolated from wistar female retired rats by portal vein perfusion method as previously described in detail (Davis et al., 1988). In brief (see also Fig. 4; p. 21), initially, phosphate-buffered saline at 37°C was perfused at 15 ml/min for 10 min to blanch the liver. This was followed by 0.05% collagenase (Wako Pure Chemical Inc., Tokyo) perfusion at 5 ml/min for 20 min at 37°C. The liver was removed and minced in serum-free DMEM (Nissui Pharmaceutical Co., Tokyo). The cell suspension was then filtered through sterile gauze and centrifuged at 50×g for 3 min to remove hepatocytes and debris. The suspension after removal of sediments containing nonparenchymal cells was layered over a 25% performed (20,000×g for 10 min) Percoll (Pharmacia Biotech., Inc., Sweden) gradient and centrifuged at 800×g for 30 min. The gradient centrifugation produced a top layer of yellowish white oily debris with a band of cells immediately beneath. The band mainly contained stellate cells as identified as lipid droplet-laden cells. Autofluorescence at 325 nm rapidly faded under the fluorescent microscope (Model DMIRB, Leica Co., Tokyo), while Oil Red O (Sigma, USA) stained the cells. From these analyses, stellate cells

predominated >90%. The cells displayed >95% viability from the examination with trypan blue exclusion. The cells were then washed two times and resuspended in DMEM supplemented with 10% FBS (Cansera International Inc., Canada) and plated at a density of 1.0×10^6 cells per 100-mm-tissue culture dish. Myofibroblast-like hepatic stellate cells (M-HSC) were obtained from primary stellate cells by 14-16 times passages up to 28-32 PDL on the 100 mm dishes.

Culture media and cell culture

HSC, RD, MDCK and HF-18 were grown in DMEM supplemented with 10% FBS, 60 μg/ml kanamycin sulfate and 50 ng/ml amphotericin-B. A-SMC were cultured in modified MCDB131 medium (Clonetics Corp.) supplemented with 10% FBS, 10 ng/ml recombinant epidermal growth factor, 2 ng/ml recombinant basic fibroblast growth factor, 5 μg/ml insulin, 50 μg/ml gentamaicin and 50 ng/ml amphotericin-B (designated as growth medium in this thesis). G-MC were cultured in modified MCDB131 medium containing 10% FBS, 50 μg/ml gentamaicin and 50 ng/ml amphotericin-B. A-EC were grown in EBM-2 medium (Clonetics Corp.) containing 2% FBS, 10 μg/ml hydrocortisone, 2 ng/ml recombinant basic fibroblast growth factor, 5 ng/ml insulin-like growth factor-1, 5 ng/ml vascular endothelial derived growth factor, 0.2 mM ascorbic acid 2-phosphate, 10 μg/ml heparin, 10 ng/ml recombinant epidermal growth factor, 50 μg/ml gentamaicin and 50 ng/ml amphotericin-B. All the additional factors above were purchased from Clonetics Corp. All of the cell types were maintained at 37°C under humidified 5% CO_2-95% air on the 100 mm-tissue culture dish. When the cells reached confluence, the cells were passaged at 1:4 split ratio after they were removed from the dish with a brief exposure to 0.25% trypsin (Difco)-0.02% EDTA in PBS (-) (Nissui Pharmaceutical Co.).

Preparation of lens capsule type IV collagen

Bovine lens capsules that had been isolated from bovine eyes were kindly provided by Nitta Gelatin, Osaka, or were isolated from bovine eyes purchased from Tokyo Shibaura Zoki, Tokyo. All the following procedures were carried out at 4°C. Type IV collagen from acid extract of bovine lens capsules without pepsin treatment was obtained as previously described (Muraoka et al., 1993). 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 (Sigma), 10 mM N-ethylmaleimide (Nacalai Tesque, Tokyo) 100 μM phenylmethylsulfonyl fluoride (Sigma), 1 mM pepstatin A (Sigma) and 0.02% sodium azide (Wako). The homogenate suspension was kept stirring for 2-3 days. After centrifugation at 800×g for 15 min, the supernatant was collected and precipitated by addition of NaCl to a final concentration of 1.2 M. The precipitate was dissolved in 0.5 M acetic acid and the solution was extensively dialyzed against 1 mM HCl and stored at 4°C before use. Protein concentrations were determined by the weight of the lyophilized material. The purity of the type IV collagen preparation was analyzed by SDS-PAGE (Fig. 5; p. 22). Electrophoresis on 5% SDS polyacrylamide gel was performed essentially as described by Laemmli (Laemmli, 1970).

Preparation of substrates and successive cell culture

Type I collagen was obtained from acid extract of rat tail tendon by previously reported method (Yamato et al., 1992). Preparation and successive culture on type I collagen gel were performed as previously described (Yamato et al., 1992). Briefly, 6 volumes of type I collagen solution (3 mg total protein/ml) was mixed with 3 volumes of 3×concentrated-culture media 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 with 5% CO_2-95% air. The medium was replaced every 3 days. Preparation of type IV collagen gel was described as previously reported (Nakazato et al., 1996). 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 dish was incubated at 4°C for at least 5 days for gel formation with sufficient rigidity to accommodate cells on top of the gel. After replacing the buffer with culture media, 1 ml of cell suspension containing 7.2×10^3 cells was gently plated on each gel and the cells were grown at 37°C with 5% CO_2-95% air. The medium was replaced every 3 days. Occasionally type IV collagen solution formed a fragile and immature gel when the solution was preserved for a prolonged period. The type IV collagen gel with insufficient rigidity was also used as a substrate whether the gel rigidity affects cellular behaviors. 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 up at 25°C. The wells were washed with PBS (-), followed by further washing with culture medium. For 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 on the dish surface at 37°C for 2h. Then, the wells were washed with PBS (-), followed by further washing either with growth medium (for M-SMC) or with DMEM containing 10% FBS (for HF-18). Cell morphology was observed with a phase-contrast microscope (Model DMIRB, Leica Co., Tokyo) at 100× magnification.

Cell proliferation assay

Quantification of cell proliferation was performed as previously described (Sakata et al., 1990). In brief, the cells cultured on gels of type I collagen and type IV collagen were washed with PBS (-) and then they were treated with 500 μl of 0.2% bacterial collagenase (Wako) for suspending the cells containing 1 mM $CaCl_2$ in PBS (-) at 37°C for 2 h. The cells cultured on plastic dishes or the 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(-). Liberated cell numbers were counted on day 2, 5 and 10 in triplicate wells with a Coulter counter (Model Z-1, Coulter Co., Tokyo).

Immunocytochemistry

M-SMC cultured on the dishes coated with type IV collagen solution, type I collagen gel or rigid type IV collagen gel for 3 days or 7 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. They were then preincubated with 0.2% BSA in PBS (-) at 25°C for 30 min for blocking nonspecific binding and incubated with a 1 : 500 dilution of anti-smooth muscle myosin heavy chain monoclonal antibody (Clone hSM-V, Sigma Co., USA), anti-human β1 integrin monoclonal antibody (MAB 1977, Chemicon Int. Inc., USA) and a 1 : 400 dilution of anti-α-smooth muscle actin monoclonal antibody (Clone 1A4, 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 with a confocal laser scanning microscope (Model TCSNT, Leica Co., Tokyo) in the fluorescence mode at 400 × magnification. Non-immune mouse IgG (ICN Pharmaceuticals, Inc., USA) was used in place of the specific antibodies against smooth muscle myosin heavy chain, human β1 integrin and α-smooth muscle actin as a control.

Endothelin-1 treatment

M-SMC were cultured on 24 well Falcon dish 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., Inc., Osaka) by addition to the culture system and observed with the phase-contrast microscope at 37 °C for 15 min. Photomicrographs were taken every 1 min at 100× magnification. The cells without the addition of endothelin-1 were used as control.

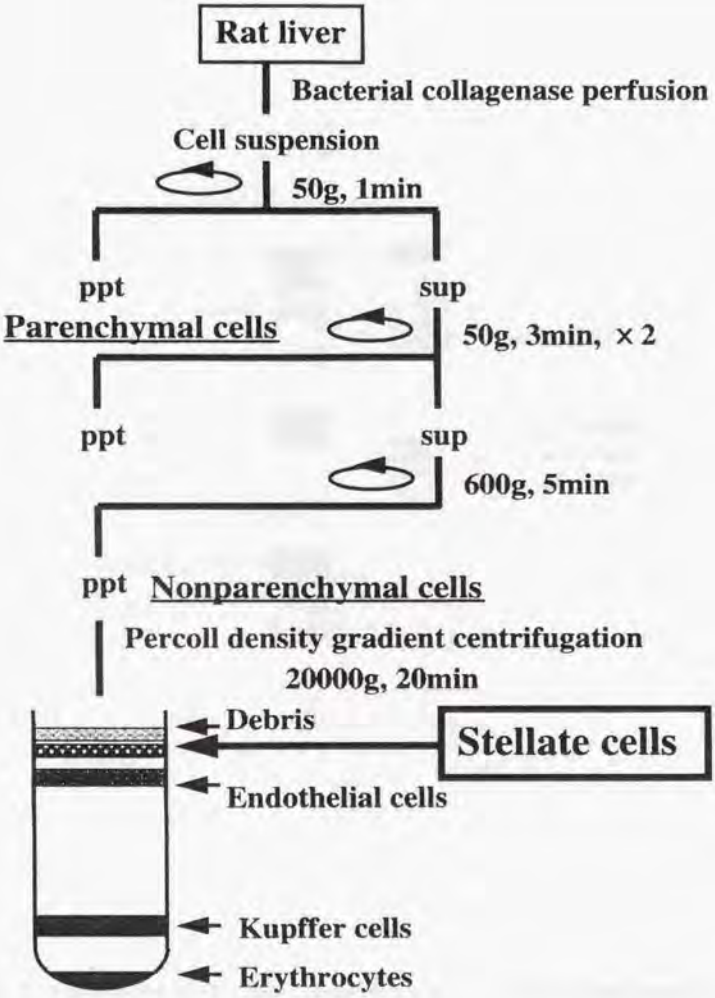


Fig. 4. Procedure for isolation of hepatic stellate cells

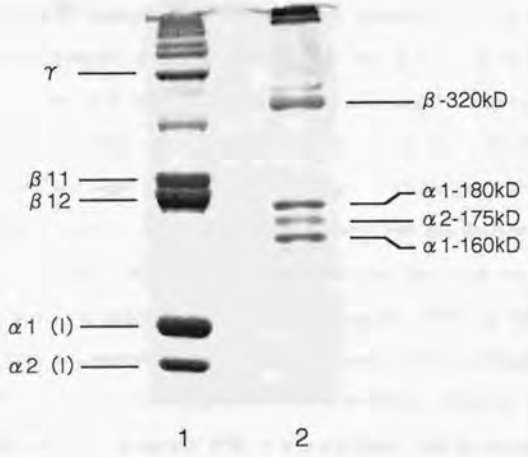


Fig. 5. Analysis of type IV collagen preparation isolated from bovine lens capsules by SDS-PAGE. Type IV collagen preparation was subjected to electrophoresis on 5% SDS polyacrylamide gel with reduction and proteins were stained with Coomassie Brilliant Blue R-250 (lane 2). Type I collagen from rat tail tendon (lane 1) was used as a molecular weight standard.

Chapter I

Specific effects of type IV collagen gel used as cell culture substrate on the morphology and proliferation of rat hepatic stellate cells in primary culture

Summary

The effects of type IV collagen gel used as cell culture substrate were examined on the shape and growth of rat hepatic stellate cells (HSC) in primary culture and compared with those of the dish coated with type IV collagen solution or type I collagen gel. HSC cultured on the type IV collagen gel showed quite a unique shape distinct from those cultured on the type IV collagen-coated dish and the type I collagen gel; Fibroblast-like bipolar cell shape was shown on the type IV collagen-coated dish and the type I collagen gel, while the cells cultured on the type IV collagen gel formed cell-to-cell junctions with cytoplasmic processes, resulting in multicellular organization. HSC on the type IV collagen gel stopped proliferation. On the contrary, molecular type IV collagen showed the highest proliferative activity among all the substrates examined. These results suggest that the morphology and proliferation of HSC in primary culture may be regulated by the state of extracellular environments including the type IV collagen aggregates.

Introduction

Hepatic stellate cells (HSC), which are present in the perisinusoidal space of Disse in normal liver, are reported to have a unique morphological appearance with three dimensional star-like shape, connecting with perisinusoidal endothelial cells and hepatocytes (Wake, 1980; 1988; 1995). It has been described that the proliferation of HSC in normal liver is little (Geerts et al., 1994). However, the cells convert their features from quiescent to activated phenotype during chronic hepatitis. In pathological states such as liver fibrosis, the cells lose retinoids, rapidly proliferate and deposit fibrillar

collagens (Davis et al., 1987b; Milani et al., 1989; Geerts et al., 1991). The cell shape changes from star-like shape to fibroblast-like shape (Mc Gee et al., 1972; Minato et al., 1983). The changes also occur *in vitro* culture. Previous studies using the dish coated with or without coated with ECM proteins indicated that HSC proliferated and secrete excessive ECM proteins (Davis et al., 1987a; 1988; Friedman et al., 1989; Senoo et al., 1994; Hirose et al., 1997). The influence of the cell environments including extracellular factors on the alteration of HSC phenotypes has not been clear. Previous immunohistochemical analyses indicated that the HSC was in a close contact with the type IV collagen aggregates in sinusoids (Yoshida et al., 1997), suggesting that at least from the location, type IV collagen in a aggregate form could influence the regulation of the HSC proliferation and morphology, if the type IV collagen aggregate has a potential of affecting HSC.

In Chapter I, therefore I examined the effects of the type IV collagen gel rigid enough to accommodate cells on top of the gel on the cellular morphology and proliferation of the HSC in primary culture by using the gel as cell culture substrate.

Results

Morphology of rat hepatic stellate cells in primary culture

Isolated HSC contained the vitamin A lipid droplets, which are characteristic for the HSC cytoplasm as shown in Fig. I-1. The isolated HSC displayed similar morphologies with bipolar shape, when they were cultured on plastic dish, type I collagen or type IV collagen-coated dish and type I collagen gel at culture day 5 (Fig I-2a, b). By contrast, on type IV collagen gel, each stellate cell elongated extremely until day 3. Thereafter, continuation of cell culture resulted in the formation of cell-to-cell junctions through the tips of the process. Finally, polygonal meshwork structure of cells was obtained at culture day 5 (Fig. I-2c). The morphology of HSC cultured on the type IV collagen gel appeared to be closest to those of the stellate cells in vivo according to Dr. Wake, K. (Fig. I-4).

Proliferation of rat hepatic stellate cells in culture

Fig. 1-3 showed the growth curves of primary cultures of HSC that were cultured on various substrates for 10 days. Numbers of cells adhered were essentially the same among the different substrates; the dishes with or without coating of type I collagen solution, type IV collagen solution, the type I collagen gel or type IV collagen gel. However, cell numbers after prolonged culture increased several times of the initial on all the culture substrates except that of the cells cultured on the type IV collagen gel. Type IV collagen gel has strong repression on HSC proliferation. Previous reports showed laminin or Matrigel that comprises basement membrane components plus some growth factors had a potency at the initial culture period of repressing HSC proliferation (Friedman et al., 1989). However, no effective substrate for the HSC was reported to repress the cell growth in culture. The type IV collagen gel is one of the candidate substrates for keeping the HSC quiescent. Upon releasing the cells from the substrate by degradation of type IV collagen with bacterial collagenase, the HSC began to grow after the change of cell shape back to spherical, followed by adhesion and spreading. This indicates that the cells cultured on the type IV collagen gel was most likely to be living without proliferation.

Discussion

Cellular functions of HSC are greatly influenced by extracellular environments. Cell shape and proliferation are fundamental functions of the HSC, since these phenotypes are apparently different between in a physiological state and in a pathological state (Minato et al., 1983; Geerts et al., 1989; 1994). Attempts to maintain the HSC functions have been done in fail even by using primary culture system. In this aspect, the type IV collagen gel is the most potent repression activity against HSC proliferation and thus it may raise a new possibility for maintaining the differentiated phenotypes. On the type IV collagen gel, the HSC elongated extremely and finally formed the cell-to-cell junction through the

elongated processes. Of particular interest is this phenomenon that the type IV collagen gel induced cellular junctions of the HSC. The junction formation of mesencymal cells is not often reported in comparison with endothelial and epithelial cells. In normal liver, as shown by Dr. Wake, K., the former Professor of Tokyo Medical and Dental University, stellate cells spread and extend processes three-dimensionally and appear to connect each other (Fig. I-4). Moreover, recent study revealed that anti-type IV collagen antibody strongly reacted with sinusoid, which did not contain apparently the basal lamina structure (Yoshida et al., 1997). The information suggests that the shape of the cells cultured on the type IV collagen gel may reflect the *in vivo* morphology of quiescent cells.

The proliferation of the primary HSC was totally repressed on the type IV collagen gel for more than 10 days. Among ECM components in contact with HSC *in vivo*, type IV collagen was the first component that might directly regulate the cell proliferation. By contrast, the dish coated with the type IV collagen solution showed the highest proliferative activity, implying that the proliferation of HSC is strongly affected by the state of type IV collagen that it forms aggregates or not. As shown in the HSC cultured on the dishes coated with the type IV collagen solution, the HSC obtained a high proliferative potential are called myofibroblasts which correspondingly found in the liver tissues in the pathological state (Kent et al., 1976; Geerts et al., 1989; Maher et al., 1989). In Chapter II, I examine the effects of type IV collagen gel on the cell shape and growth of myofibroblast-like hepatic stellate cells.

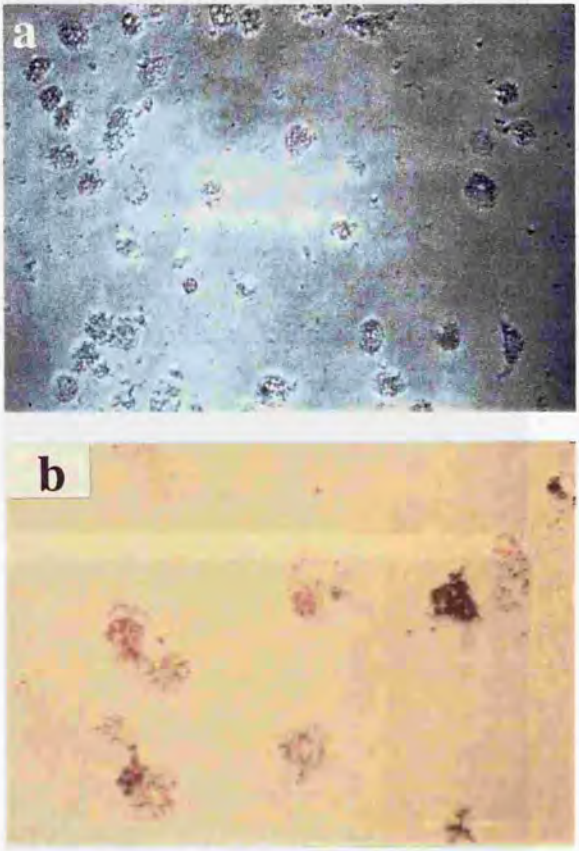


Fig. I-1. Hepatic stellate cells isolated from rat liver. Isolated HSC were observed under the phase-contrast microscope. All of the cells contained the lipid droplets in the cytoplasm (a). Cytoplasmic droplets were stained with Oil red-O (b).

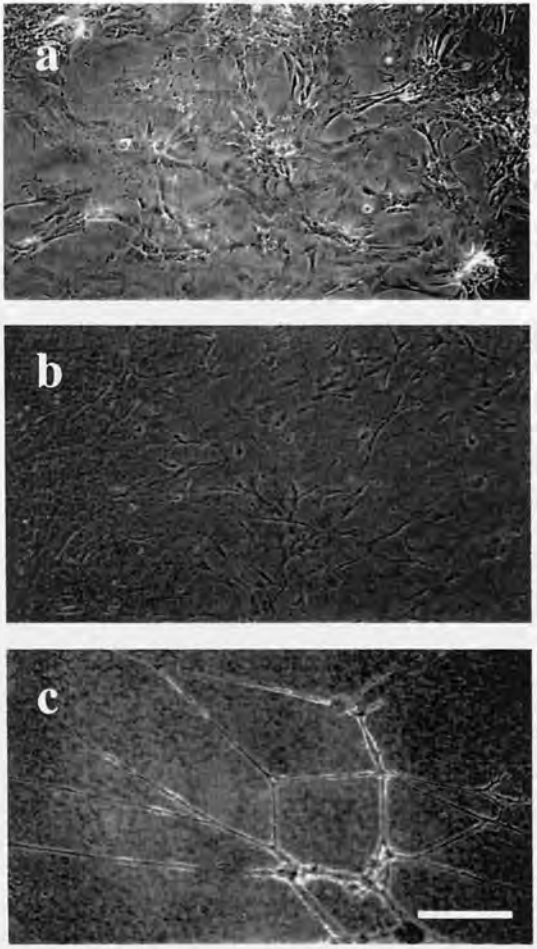


Fig. I-2. Morphology of hepatic stellate cells in primary culture. Primary cultures of HSC were grown on type IV collagen-coated dish (a), type I collagen gel (b) or type IV collagen gel (c) in DMEM supplemented with 10% FBS. At culture day 5, morphology of the cells was observed with the phase-contrast microscope and photomicrographed. Bar; 100μm.

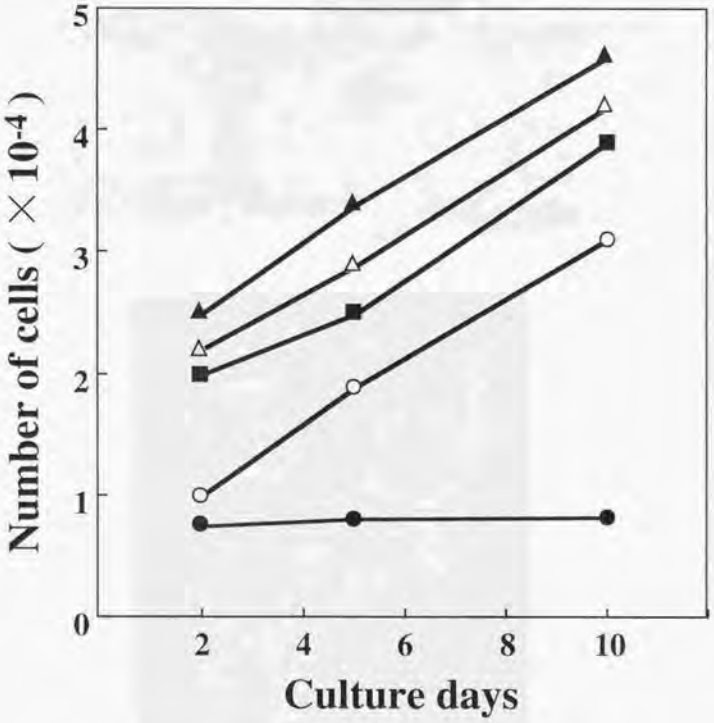
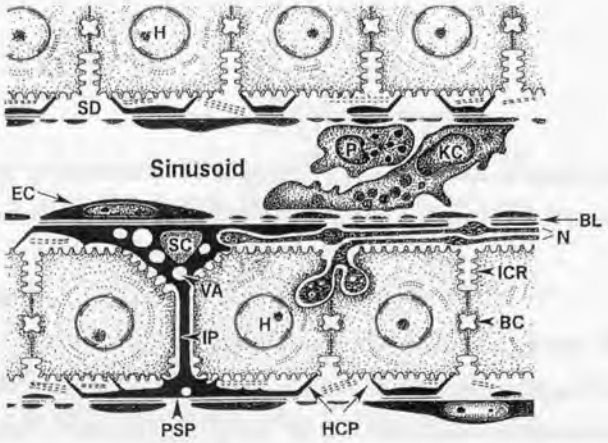
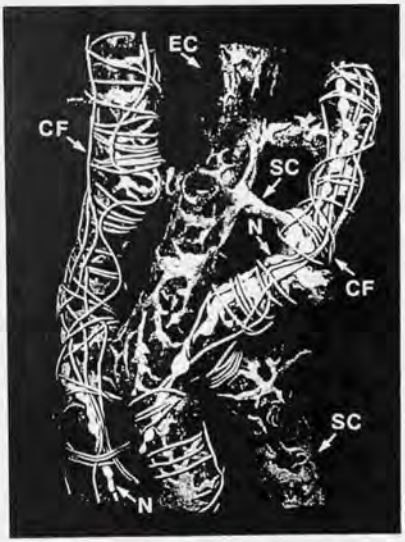


Fig. I-3. Growth curves of hepatic stellate cells in primary culture. Primary cultures of HSC were grown on plastic dish (■), type I collagen-coated dish (△), type IV collagen-coated dish (▲), type I collagen gel (○) or type IV collagen gel (●) in DMEM supplemented with 10% FBS for 10 days. The cells were initially plated at a cell density of 7.2×10^3/ well on 24 well culture dish. The medium was renewed every 3 days. The number of cells was counted on day 2, 5 and 10 with the Coulter counter after the cells were removed from the dish. Data represent the mean number of the cells of three determinations in three separate experiments.





A model of the sinusoidal wall. showing endthelial cell (EC), stellate cell (SC), collagen fibers (CF) and nerve fibers (N).

(K.Wake Cells of the Hepatic Sinusoid 5 (1995) pp.242)

Fig. 1-4. Schematic models of perisinusoidal cells in liver

Chapter II

Potent growth-inhibition by type IV collagen gel used as culture substrate of the hepatic stellate cells that once acquired a high growth potential

Summary

As shown in Chapter I, primary HSC formed intercellular junctions with extended cytoplasmic processes in culture on the type IV collagen gel, resulting in formation of a mesh-like multicellular organization. The cells showed little proliferative activity for at least 10 days on the type IV collagen gel. By contrast, the cells assumed the elongated bipolar cell shapes and showed high proliferative activity in culture on the dishes coated with type I collagen solution and type IV collagen solution not in a gel form or on type I collagen gel. It is well known that HSC loses *in vivo* features upon *in vitro* culture particularly after many passages. Phenotypically changed cells might correspond to what are called myofibroblasts found in fibrotic or cirrhotic state of the liver tissue. The myofibroblast-like hepatic stellate cells (designated as M-HSC in short in this thesis) obtained by repeated passages up to 28 or 32 PDL were cultured on the dishes coated with type I collagen solution, type IV collagen solution, the type I collagen gel or the type IV collagen gel. The M-HSC represented the same bipolar shape as the primary culture cells, while on the type IV collagen gel, they behaved similarly to the primary culture cells, forming multicellular network with their processes connecting each other. Furthermore, the M-HSC proliferated only slightly on the type IV collagen gel, quite a contrast with the cells cultured on the collagen-coated dishes or on the type I collagen gel. The results suggest that appropriate ECM environment comprising the type IV collagen gel provides the M-HSC with a unique extracellular signal involved in the regulation of cell morphology and proliferation.

Introduction

It is widely accepted that animal tissue cells lose their *in vivo* morphological and functional features upon *in vitro* culture. This is also the case with HSC. HSC often morphologically change to be fibroblastic, acquire strong proliferative activity and produce increased fibrillar collagen after repeated passages or prolonged culture *in vitro* (Friedman et al., 1989; Geerts et al., 1989; 1994). The cells can be taken as the myofibroblasts correspondingly found in the liver tissues under pathological states (Geerts et al., 1989; Maher et al., 1989). A strong argument for the involvement of hepatic stellate cell-derived myofibroblastic cells has been postulated in liver fibrosis or cirrhosis. That is, pathological view of liver fibrosis or cirrhosis would be that HSC became synthetic to be proliferative, migrating and producing collagenous fibrils. Thus, it would be very important to control the growth of myofibroblast-like hepatic stellate cells (M-HSC) in order to develop a new therapy for liver fibrosis. However, no therapeutic way has been reported for repressing the progression of hepatic stellate cell-derived myofibroblasts.

In Chapter I, I showed that the rigid type IV collagen gel totally repressed the growth of primary HSC in culture. Here in Chapter II, I examined the type IV collagen gel with sufficient rigidity on morphological features and proliferative activity of HSC with a high growth potential after the cells were repeatedly passaged on plastic dishes. This may provide the information as to how far the effects of the rigid type IV collagen gel extend to myofibroblast-like hepatic stellate cells.

Results

Proliferation of the rat hepatic stellate cells that acquired a high proliferative potential through many passages in culture

The rat HSC were cultured on the bare plastic dish from primary and underwent serial passages up to 28 or 32 PDL. The cells began to grow at an earlier timing of culture.

Such cells that acquired a high proliferative potential were defined as myofibroblast-like hepatic stellate cells (M-HSC) in this thesis. As shown in Fig. II-1, the M-HSC proliferated more slowly on type IV collagen gel than on type I collagen or on plastic dishes. Though the cells slightly grew for 10 days, the type IV collagen gel significantly suppressed the growth of M-HSC. It was intriguing that the type IV collagen molecules induced mHSC growth to the highest number of cells among the substrates examined, suggesting that M-HSC retains the feature of primary HSC susceptible to the state of the type IV collagen.

Morphology of myofibroblast-like stellate cells

Fig. II-2 showed the cell shape at culture day 5. The M-HSC with 28 PDL showed the same morphological features as the primary culture cells did: The bipolar shape on the coated with or without type I collagen solution or type IV collagen solution. On type I collagen gel, the cells more extremely elongated than on the dish. On the type IV collagen gel they behaved similarly to the primary culture cells, forming intercellular network. The HSC that had become M-HSC after repeated passages on plastic dish showed essentially the same cell shapes as the primary HSC depending on the substrates.

Discussion

In Chapter I, I presented that rat HSC of the primary culture cultured on type IV collagen gel showed distinct morphological appearance, particularly forming intercellular network, from the cells cultured on other substrate including type I collagen gel. In Chapter II, Cells similar to myofibroblasts that had acquired the proliferative potential through *in vitro* passages, became highly elongated and formed junctions through process, resulting in multicellular polygonal meshwork, and stopped proliferation upon culturing on the type IV collagen gel. The findings, to our knowledge, are the first demonstration that the proliferation of the M-HSC with high proliferative potential was totally repressed

in the particular consolidated environments.

The interaction between ECM environment and HSC appeared to preserve specificity over the repeated passages in culture, suggesting that HSC may be one of the most sensitive cell types to changes in the extracellular environments. That is, the HSC alter cell morphology and proliferative activity in response to the extracellular consolidated materials. It would be of interest to elucidate the structure of type IV collage gel responsible for the growth-repression activity.

Molecular form of the type IV collagen showed the most significant effect on the growth of M-HSC. The result might provide us with practically important cell technology to obtain a great number of the differentiated HSC. That is, in first M-HSC were grown and many numbers of cells can be obtained by culturing on the molecular type IV collagen. The dedifferentiated cells could be reversibly back to the differentiated states as contractile cells by culturing on the type IV collagen gel.

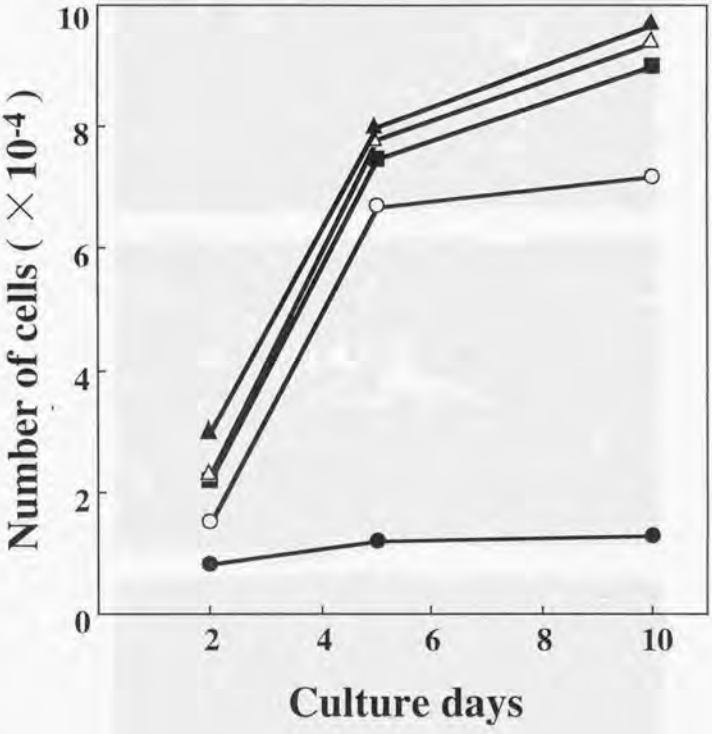


Fig. II-1. Growth curves of myofibroblast-like hepatic stellate cells. Myofibroblast-like hepatic stellate cells obtained from primary HSC by 14 times passages up to 28 PDL were cultured on plastic dish (■), type I collagen-coated dish (△), type IV collagen-coated dish (▲), type I collagen gel (○) or type IV collagen gel (●) in DMEM supplemented with 10% FBS for 10 days. The cells were plated at a initial cell density of 7.2×10^3/ well on 24 well culture dish. The medium was renewed every 3 days. The number of cells was counted on day 2, 5 and 10 with the Coulter counter after the cells were removed from the dish. Data represent the mean number of the cells of three determinations in three separate experiments.

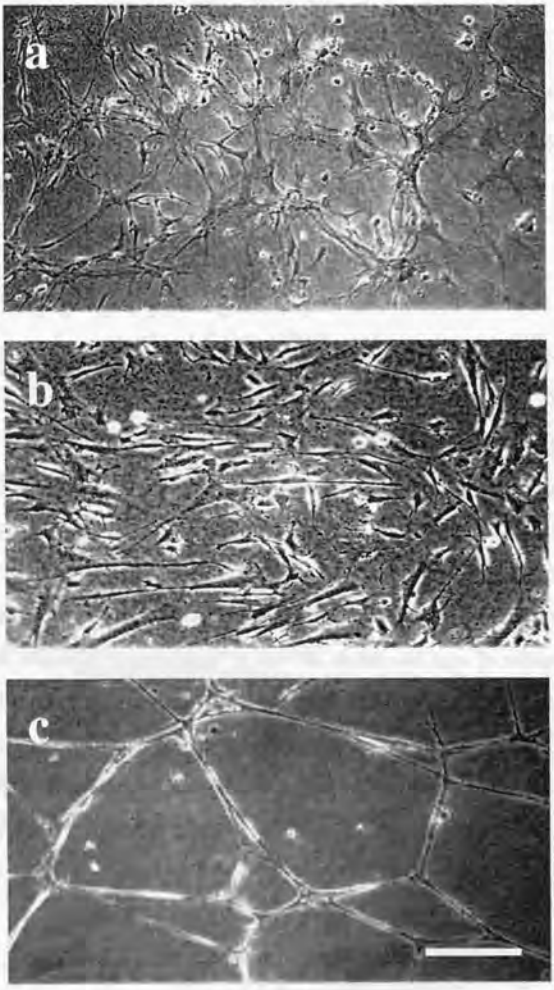


Fig. II-2. Morphology of myofibroblast-like hepatic stellate cells. Myofibroblast-like hepatic stellate cells were cultured on type IV collagen-coated dish (a), type I collagen gel (b) or type IV collagen gel (c) in DMEM supplemented with 10% FBS. At culture day 5, morphology of the cells was observed with the phase-contrast microscope and photomicrographed. Bar; 100μm.

Chapter III

Restoration of quiescent and contractile phenotype by the culture on type IV collagen gel from myofibroblast-like human aortic smooth muscle cells with a high proliferative activity

Summary

Aortic smooth muscle cells (A-SMC) undergo phenotypic transition to synthetic and proliferative state by serial passages with culturing on plastic dish particularly in the presence of serum. The fibroblast-like cells derived from A-SMC (M-SMC) may correspond to the cells called myofibroblasts in pathology. I examined the effects of type IV collagen gel used as culture substrate on the morphology and proliferation of M-SMC. The M-SMC extremely elongated in shape upon culturing on rigid type IV collagen gel and eventually formed cell-to-cell junctions with the elongated processes. In contrast, M-SMC showed a spindle-like cell shape on the dish coated with type IV collagen solution and type I collagen solution, or on type I collagen gel and fragile type IV collagen gel. The cell proliferation was totally repressed on the rigid type IV collagen gel for over 10 days, while they showed the highest proliferative activity on the dish coated with type IV collagen solution. Expression of smooth muscle myosin heavy chains, specific marker for the contractile A-SMC, was acquired by the M-SMC cultured on the rigid type IV collagen gel for 3 days, while M-SMC cultured on the type IV collagen-coated dish remained to show no expression. These results suggest that quiescent and contractile phenotype of A-SMC might be restored from M-SMC by the culture on the rigid type IV collagen gel even after they had become myofibroblastic.

Introduction

It has been reported that in atherosclerotic lesions aortic smooth muscle cells (A-SMC) change the cell differentiation states from contractile to synthetic (Thyberg et al., 1990; Ross, 1993). Phenotypes of A-SMC changed within the lesions include morphological appearance, potent proliferative activity, production of excessive fibrillar collagen, and deposition of collagen fibrils (Thyberg, 1996; Chamley-Campbell et al., 1981a), all of which are similar to fibroblasts. The observations have tempted the pathologists to refer to the smooth muscle-derived cells as myofibroblasts. Regulation of smooth muscle phenotypes is considered to be crucial in the pathogenesis of fibrosis or cirrhosis of various organs. A similar change to fibroblastic phenotype occurs in A-SMC during a prolonged period of *in vitro* primary culture and/or repeated passages with serum-containing culture medium (Thyberg, 1996; Chamley-Campbell et al., 1979; 1981b; Campbell et al., 1981). Thereby the cells may have acquired properties corresponding to those of the myofibroblasts found in the atherosclerotic lesions (Thyberg, 1996; Rekhter et al., 1995).

Cell fundamental functions including growth, maintenance of differentiation, gene expression, cell migration as well as cell attachment are affected by the surrounding extracellular matrix (ECM). The direct evidence was obtained by *in vitro* studies through analyses of the effects of substrates on cultured cells (Lin et al., 1993; Jones et al., 1993; Thoumine et al, 1995; Berthiaume et al., 1996). Functions of A-SMC were affected and thus regulated in cell culture by ECM used as consolidated culture substrates (Thyberg, 1996; Thyberg et al., 1983). Previous reports described that laminin and type IV collagen might have helped to retard the de-differentiation of cultured A-SMC for an initial period without serum (Hedin et al., 1988; Thyberg et al, 1994). However, A-SMC *in vitro* culture tended to lose the differentiated phenotypes in a longer term of culture. Particularly in the presence of serum, A-SMC become fibroblastic, regardless whether they are cultured on the dish coated with any of the ECM components. Solubilized collagenous

proteins were not exceptional (Thyberg et al., 1983; Yamamoto et al., 1993).

Isolated type I collagen can be reconstituted into fibrillar aggregates with 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 were cultured within reconstituted type I collagen gel, their phenotypes were quite similar to those of the fibroblasts (Ehrlich et al., 1986). Namely, the collagen gel was contracted by these cells (Ehrlich et al., 1986; Yamamoto et al., 1996; Bell et al., 1979; Yamato et al., 1995). The cell shape was extremely elongated, while the cell proliferation was repressed (Yamamoto et al., 1996; Nishiyama et al., 1989; Hayashi et al., 1993). Recently it was reported that fibrillar type I collagen (Koyama et al., 1996) or gel form of type I and type III collagen (Yamamoto et al., 1996) retard the A-SMC proliferation in primary cultures without serum. The findings prompted me to speculate that assembled collagenous proteins might play another different activity from the monomeric or soluble proteins.

Adachi et al. have proposed a new hypothesis that tissue collagen type distribution has a graded structure (Adachi et al., 1997a). I have recently extended the idea further so that the localization of cell types is incorporated into the tissue graded structure of collagen aggregates, particularly tissue co-localization of the type IV collagen and A-SMC. That is, A-SMC are in contact with type IV collagen in tissue, but dermal fibroblasts are not (Kino et al., 1991). I then have addressed a question whether the relationships between collagen aggregates and corresponding cell types in tissues have a biological meaning such as maintenance of the cell phenotypic differentiation.

The 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 Adachi et al. recently found that the isolated type IV collagen formed polygonal meshwork with pores of 18 nm in diameter corresponding to the skeletal structure of lamina densa (Adachi et al., 1997b).

Upon formation of the polygonal meshwork, the type IV collagen solution gelled under selected conditions of NaCl and temperature (Nakazato et al., 1996). Gel rigidity particularly depended on temperature and incubation time as well as protein concentration (Nakazato et al., 1996). Contrary to the case of type I collagen gel, the rigidity of the type IV collagen gel requires a low temperature. I here in Chapter III examine the effects of rigid type IV collagen gel used as cell culture substrate on fundamental cellular functions of myofibroblast-like A-SMC (designated as M-SMC in this report) including cell shape, cell growth, and marker protein expression in the culture with 10% FBS.

Results

Morphology of M-SMC in culture

In the presence of 10% FBS, repeated passages on plastic dish of A-SMC starting from passage 3 up to 9-13 PDL acquired a high growth activity. In this study, I refer to these cells as myofibroblast-like smooth muscle cells or M-SMC in short. Cultured M-SMC expressed α-smooth muscle actins (α-SMA) for the marker proteins of A-SMC (Fig. III-1). In contrast, HF-18, normal human dermal fibroblasts, did not express α-SMA at all (data not shown). The results revealed that the M-SMC used were derived from A-SMC. To determine whether the chemical and supramolecular structures of type I collagen and type IV collagen affect cell morphology of M-SMC, I prepared six different substrates as follows; 1) the dish coated with type I collagen solution, 2) the dish coated with type IV collagen solution (Fig. III-2a), 3) type I collagen gel (Fig. III-2b), 4) the dish coated with aggregated type IV collagen (non-gel form) (Fig. III-2c), 5) type IV collagen gel with sufficient rigidity (Fig. III-2e, f) and 6) type IV collagen gel with insufficient rigidity (Fig. III-2d). Cell morphology of the M-SMC with 9 PDL on six kinds of different substrates was observed under the phase-contrast microscope at culture day 3. The M-SMC started to spread at 2h after cultivation on all of the substrates except for type I collagen gel or type IV collagen gel with sufficient rigidity. However, spreading

of the cells on the type I collagen gel started in 5h, and then they showed similar morphology with spindle-like or elongated bipolar cell shape to the cells cultured on the dish coated with type IV collagen solution (Fig. III-2a, b), the dish coated with aggregated type IV collagen (Fig. III-2c), bare plastic dish, and type I collagen-coated dish (data not shown). Culturing M-SMC on the rigid type IV collagen gel caused the cells to be retarded in initiating to spread and elongate as late as the cells cultured on type I collagen gel. Once the cells started elongation, it continued for about 2 days. Thereafter, extremely elongate cell terminals could not be distinguished due to apparent formation of cell-to-cell junctions with adjacent elongated cells. The cell-to-cell junctions predominated over the entire cells cultivated, eventually giving rise to mesh-like multicellular organization as shown in Fig. III-2e. The meshwork formation was completed by culture day 3 and maintained for more than 14 days (Fig. III-2f). The multicellular meshwork structure began to collapse upon treatment with bacterial collagenase. The cells detached from each other to be rounded finally. However, when the rounded cells were collected and seeded again on the bare plastic dish, they re-started to take spindle-like shape with a high proliferative activity. These indicate that the repression of M-SMC proliferation on the rigid type IV collagen gel was reversible.

It takes a longer time of incubation for type IV collagen solution to gel with a rigidity comparable type I collagen gel. Fragile gels were obtained when the incubation at 4°C was shortened. Thus when cells were placed on the type IV collagen gel with insufficient rigidity, they sometimes sedimented down to bottom of the gel. Such cells in contact with the dish began to spread sooner or later. Eventually, they showed a similar cell shape to those cultured on the dish coated with the type IV collagen solution (Fig. III-2d). The results suggest that morphology of M-SMC was strongly affected by whether the type IV collagen aggregates were in a gel form or not.

Proliferation of M-SMC in culture

M-SMC which underwent repeated passages on plastic dish up to 9 PDL acquired a high growth potential as shown in Fig. III-3. The proliferation of the M-SMC was determined in the presence of 10% FBS on different substrates. Cell attachment is a prerequisite for the cell growth. The cell numbers attached to the substrate were not different among the substrates examined. M-SMC cultured on type I collagen gel did not initiate cell growth until day 2. However, once the cell growth started, the growth rate on the type I collagen gel was as high as that of the cells on the plastic dish (Fig. III-3). The highest cell number at culture day 10 was seen for the M-SMC cultured on the dish coated with the type IV collagen solution (Fig. III-3). On the other hand, the number of M-SMC did not increase at all over 21 days on the rigid type IV collagen gel (Fig. III-3). 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 dish (data not shown).

Expression of the marker proteins for contractile stage of 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) (Kocher et al., 1986; Campbell et al., 1989), smooth muscle myosin heavy chains (SMH) (Kuro-o et al., 1989; Birukov et al., 1993) and caldesmon (Shirinsky et al., 1991) were expressed or not. Among these marker proteins, SMH is generally assumed to be the most specific and reliable marker for contractile stage of A-SMC (Birukov et al., 1993). For the analysis, the antibody that does not cross-react with skeletal, cardiac, or non-muscle myosins is required (Longtine et al., 1985). Such anti-SMH monoclonal antibodies were used for an immunocytochemical analysis of the expression of SMH in M-SMC with 13 PDL cultured on the different substrates. SMH was strongly stained in the M-SMC

cultured on the rigid type IV collagen gel for 3 days (Fig. III-4c). On the other hand, M-SMC displayed little expression of SMH in the M-SMC cultured on the dish coated with type IV collagen solution (Fig. III-4a). In the cells cultured on the type I collagen gel, SMH seemed to be expressed, but only slightly (Fig. III-4b). At culture day 7 on the type IV collagen-coated dish, the cells did not show immunoreactivity at all (Fig. III-3d). Diffuse and weak SMH staining was noted in SMC cultured on the type I collagen gel (Fig. III-4e). In contrast, strong immunostaining revealed the maintenance of a marked expression of SMH in the M-SMC cultured on the rigid type IV collagen gel at culture day 7 (Fig. III-4f). Background fluorescence was null in the control specimens from culture days 3 and 7, when they were 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 with 13 PDL in culture at day 3 whether the M-SMC maintains or restores the contractility by the drug. Upon addition of endothelin-1, only the M-SMC cultured on the rigid type IV collagen gel slightly and slowly changed the morphology in a form of multicellular network (Fig. III-5). The 15 consecutive photographs taken every one minute showed a slight but marked movement of the portions of the cell meshwork. The movements of the cells were not seen after 15 min. The change of morphology may correspond to the contraction of M-SMC (Fig. III-5). Contraction was not observed for the cells cultured on the dishes coated with type IV collagen solution and type I collagen solution or on type I collagen gel (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 the dish coated with type IV collagen solution, type I collagen gel or rigid type IV collagen gel were similar to those of M-SMC as observed under the

phase-contrast microscope. HF-18 represented elongate or spindle shape on all the substrates. HF-18 cultured on the rigid type IV collagen gel also formed multicellular meshwork at culture day 2. However, as HF-18 cells started to grow on the rigid type IV collagen gel, the meshwork structure appeared to dissipate. At culture day 5, HF-18 and M-SMC showed quite different shape on the rigid type IV collagen gel as shown in Fig. III-6c (Compare with Fig. III-2e). HF-18 did not keep intercellular junctions on the rigid type IV collagen gel. HF-18 cultured on the dish coated with type IV collagen solution (Fig. III-6a) or type I collagen gel (Fig. III-6b) showed similar morphologies to the M-SMC cultured on these substrates.

Proliferation of HF-18 in culture

The proliferation of cultured HF-18 was examined on the different substrates. The attachment of HF-18 was not different among the substrates used. Though HF-18 showed a 2 day lag period before starting to grow on type I collagen gel and rigid type IV collagen gel, the growth rate of HF-18 was almost the same on all the substrates (Fig. III-7). HF-18 grew on the rigid type IV collagen gel to the contrary of M-SMC (Fig. III-7).

Discussion

It is well known that A-SMC recovered from artery tissues as primary contractile cells become eventually synthetic through repeated passages *in vitro* cultivation particularly in the presence of serum (Thyberg, 1996; Chamley-Campbell et al, 1979; Chamley-Campbell et al, 1981; Thyberg et al., 1983). The synthetic A-SMC thus obtained are named M-SMC in this report. At an initial stage of culture (up to culture day 2), essentially no difference in morphological characteristics was seen for M-SMC and HF-18. However, morphological characteristics were noted for the M-SMC cultured on the rigid type IV collagen gel at day 5 distinct from the M-SMC cultured on the other substrates and from the HF-18 cultured on the rigid type IV collagen gel (Fig. III-2, Fig.

III-6). These 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 formation of cell-to-cell junctions. The multi-cellular structure at a sparse density of cells was a cell network as a whole (Fig. III-2e, f). A possibly corresponding case *in vivo* could be found in small blood vessels where pericytes are rather sparsely distributed underneath the endothelial cells (Hirschi et al., 1996).

Culture on the dish coated with type IV collagen solution facilitated the proliferation of M-SMC with serum (Fig. III-3) in comparison with that on a bare plastic dish. To our surprise, however, the rigid type IV collagen gel showed entirely an opposite effect. Namely, the M-SMC proliferation was repressed. The M-SMC was totally quiescent up to 21 days of culture on the rigid type IV collagen gel even in the presence of 10% FBS (Fig. III-3). As stated above, the dish coated with the type IV collagen solution showed a growth stimulating effect similar to, if not stronger than, the dish without coating or the dish coated with the type I collagen solution (Fig. III-3). Correspondingly, the cell morphology was similar among the M-SMC on all the substrates except the cells on the rigid type IV collagen gel (Fig. III-2). These indicate that the protein structure of type IV collagen could not be alone responsible for the apparent effect of the rigid type IV collagen gel on the behaviors of M-SMC. Instead, a gel form with sufficient rigidity of the type IV collagen was important. The contact surface areas between fine filaments comprising the gel and cells are restricted. Thus, most of the basal surface of the cells may be open for diffusible substances. The study on the immunocytochemical localization of β1 integrin showed a diffuse distribution on the entire surface of the basal surface regardless of the substrates (Fig. III-8), as was seen for diffuse or spotted localization of β1 integrin on the fibroblasts cultured within type I collagen gel (Yamato et al., 1998).

α-SMA and calponin have been known as protein markers for the differentiated A-SMC in the contractile state (Kocher et al., 1986; Campbell et al., 1989; Birukov et al.,

1991). However, specificity of α-SMA for the contractile state of A-SMC has been suspected recently, because the proteins are expressed in A-SMC in the synthetic state or in non-muscle cells (Woodcock-Mitchell et al., 1988). On the other hand, SMH seems to be specific for A-SMC in the contractile state (Kuro-o et al., 1989; Birukov et al., 1993). Immunocytochemical analyses with anti-SMH antibody revealed that the rigid type IV collagen gel induced the expression of SMH in the M-SMC by culture day 3 (Fig. III-4c) and kept it up to culture day 7 (Fig. III-4f), suggesting for a possible restoration from the synthetic and proliferative M-SMC back to the contractile state by culturing on the rigid type IV collagen gel. In contrast, the cells on the type IV collagen-coated surface showed null immunofluorescence (Fig. III-4d), suggesting that the synthetic cells remained as they had been seeded.

Contractility is one of the most basic functions of A-SMC. M-SMC which had lost contractile ability might have reversibly regained the contractility upon culturing on the rigid type IV collagen gel (Fig. III-5), implying that the M-SMC had re-differentiated on the rigid type IV collagen gel.

What characteristics of the rigid type IV collagen gel is responsible for the specific effect on M-SMC? The gel rigidity or elasticity may in part be important. Nakazato et al. previously reported that the rigidity of type IV collagen gel depended greatly on protein concentration, NaCl concentration, temperature of incubation and length of incubation time for the gel formation (Nakazato et al., 1996; Figures are shown in Fig. 2; p. 12, 13). Thus, when the incubation time for gel formation was shortened, insufficiently rigid gel was formed. As shown in Fig. III-2a and III-2d, the cells cultured on fragile type IV collagen gel showed a similar morphology to those cultured on the dish coated with type IV collagen solution, suggesting that mechanical rigidity of a gel might be an important factor.

How far is the effect of the rigid type IV collagen gel extended to other differentiated cells? The rigid type IV collagen gel showed similar effects on myofibroblast-like kidney

mesangial cells and hepatic stellate cells (Fig. II-1, Fig. II-2, Fig. 6, Fig. 7). That is, the rigid type IV collagen gel repressed the growth and induced the cellular morphologies to form multicellular meshwork. The mesangial cells, hepatic stellate cells and A-SMC have several common characteristics such as contractility in response to the external stimuli, production of collagenous proteins, tissue localization as the site underneath the endothelial or epithelial cells, and phenotypic transformation into fibroblast-like cells (Singhal et al., 1986; Sakamoto et al., 1993; Nerlich et al., 1993; Geerts et al., 1989; Milani et al., 1989; Wake, 1980).

M-SMC share many functional and morphological analogies with fibroblasts (Desmouliére et al., 1995). M-SMC contract collagen gel when cultured within the type I collagen gel as fibroblasts do. The cells that contribute to pathogenesis of fibrosis or sclerosis in the fibrotic or cirrhotic tissues by depositing fibrous materials are not well distinguished between fibroblasts and myofibroblasts. It is not easy to distinguish M-SMC from the fibroblasts *in vitro* culture and *in vivo*. However, as seen in this report, dermal fibroblasts showed distinct behaviors from M-SMC in the culture with rigid type IV collagen gel. The fibroblasts proliferated and showed bipolar shape without cell-to-cell junctions at a later stage of culture on the rigid type IV collagen gel (Fig. III-6, Fig. III-7). Thus, with the use of the rigid type IV collagen gel, 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 of the cell receptor-mediated signal transduction for the rigid type IV collagen gel. The detail remains to be elucidated.

In conclusion of Chapter III, the present study demonstrated some characteristic effects of the rigid type IV collagen gel on the behaviors of M-SMC: 1) The multicellular formation with cell-to-cell connection, 2) the growth suppression, 3) the expression of contractile marker proteins, and 4) the contractility of A-SMC. The results might raise an entirely new possibility that the rigid type IV collagen gel may help myofibroblast-like cells restore the quiescent and contractile A-SMC.

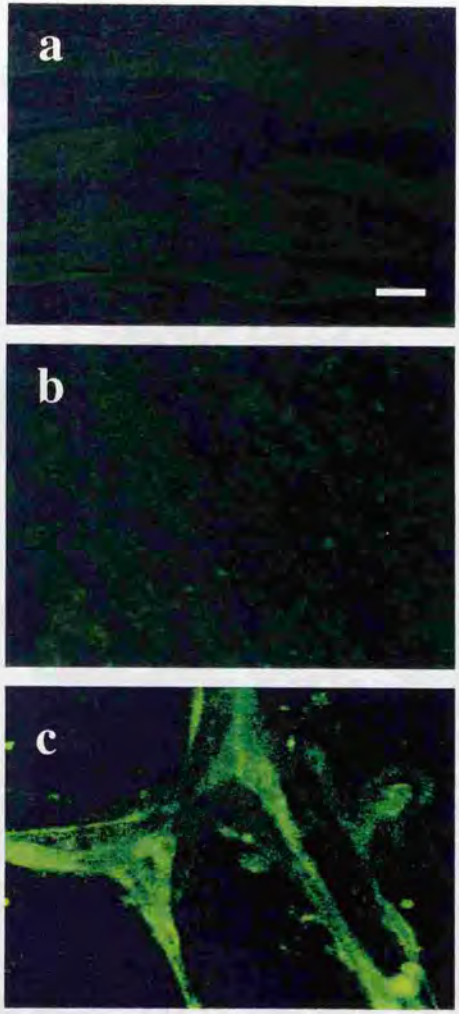


Fig. III-1. α-SMA staining of M-SMC cultured on different substrates. M-SMC obtained from A-SMC through 3 times passages up to 9 PDL were cultured on type IV collagen-coated dish (a), type I collagen gel (b) or type IV collagen gel (c). At culture day 3, the cells were fixed and permeabilized at 25°C. Then they were incubated with anti-α-smooth muscle actin monoclonal antibody at 4°C for 18 h. After exposure to FITC-prelabeled goat anti-mouse IgG, the cells were observed with the confocal laser scanning microscope and photomicrographed. No specific staining was observed in control cells (data not shown). Bar; 10μm.

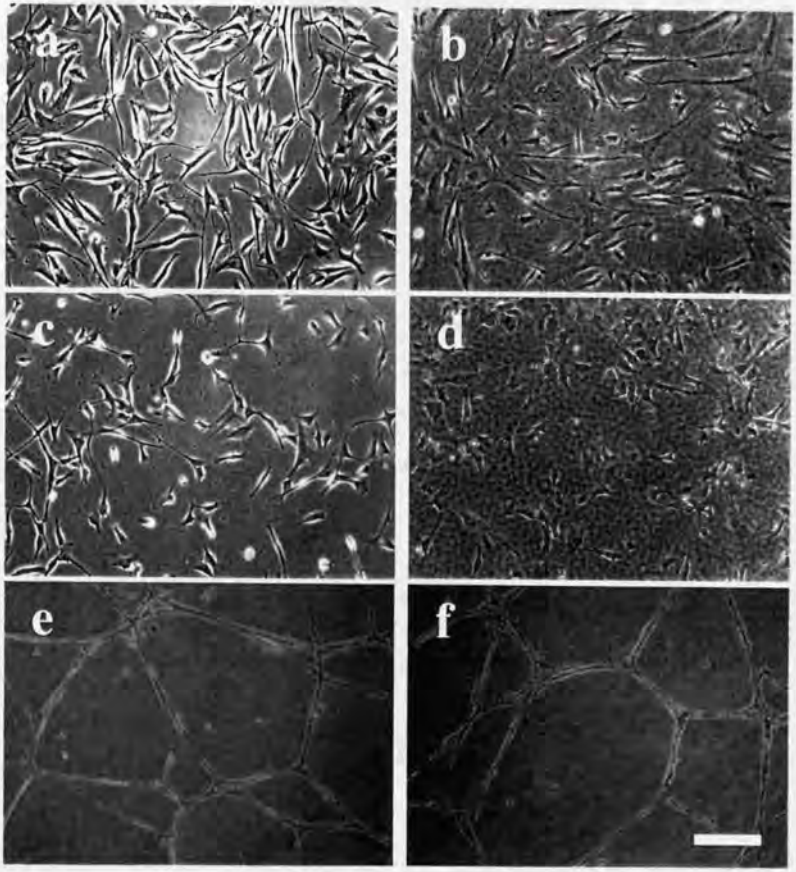


Fig. III-2. Morphology of M-SMC cultured on different substrates. M-SMC obtained from A-SMC through 3 times passages up to 9 PDL were cultured on type IV collagen-coated dish (a), type I collagen gel (b), aggregated type IV collagen-coated dish (c), immature type IV collagen gel (d) or type IV collagen gel (e, f). At culture day 3 (a-e) or day 14 (f), morphology of the cells was observed with the phase-contrast microscope and photomicrographed. Bar; 100μm.

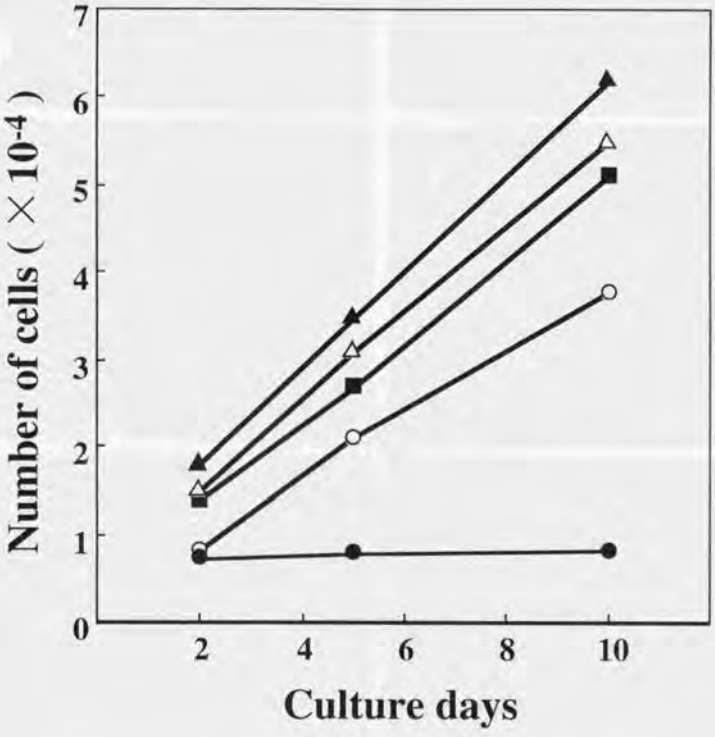


Fig. III-3. Growth curves of M-SMC cultured on different substrates. M-SMC obtained from A-SMC through 3 times passages up to 9 PDL were cultured on plastic dish (■), type I collagen-coated dish (△), type IV collagen-coated dish (▲), type I collagen gel (○) or type IV collagen gel (●) in the medium as described in Experimental Procedures for 10 days. The cells were initially plated at a cell density of 7.2×10^3/ well on 24 well culture dish. The medium was renewed every 3 days. The number of cells was counted on day 2, 5 and 10 with the Coulter counter after the cells were removed from the dish. Data represent the mean of three determinations in three separate experiments.

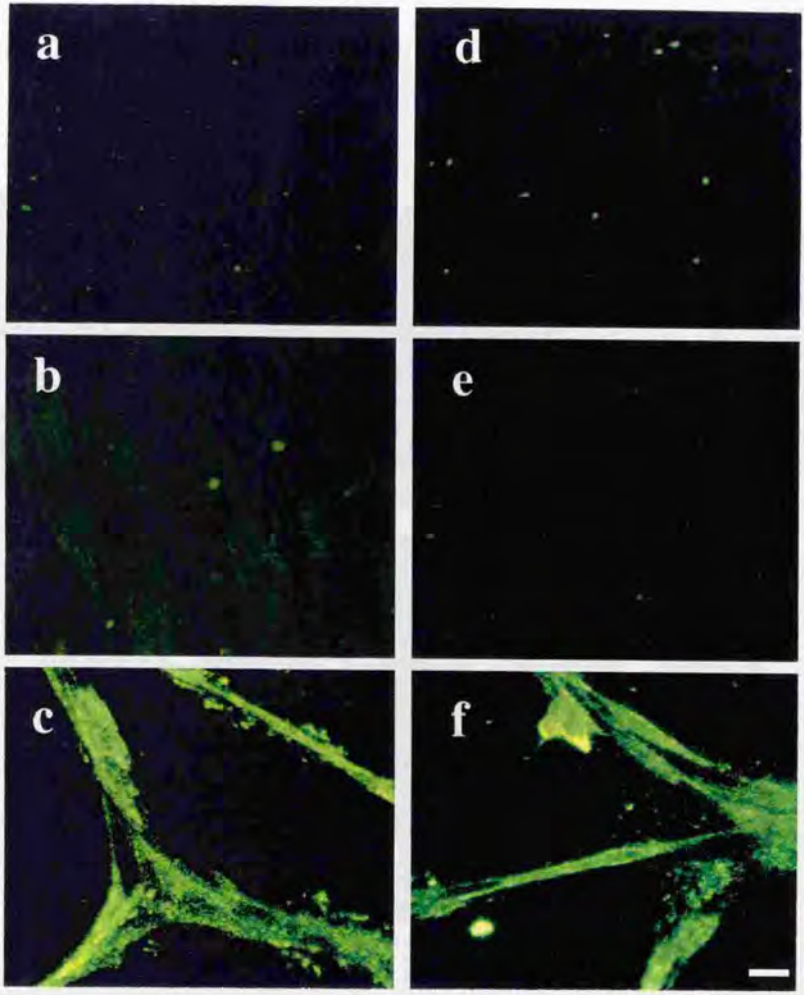


Fig. III-4. Expression of SMH in M-SMC cultured on type IV collagen-coated dish, type I collagen gel or type IV collagen gel. M-SMC obtained from A-SMC through 5 times passages up to 13 PDL were cultured on type IV collagen-coated dish (a, d), type I collagen gel (b, e) or type IV collagen gel (c, f) for 3 days (a-c) or 7 days (d-f). The cells were fixed and permeabilized at 25°C. Then they were incubated with anti-SMH monoclonal antibody at 4°C for 18 h. After exposure to FITC-prelabeled goat anti-mouse IgG, the cells were observed with the confocal laser scanning microscope and photomicrographed. Bar; 10μm.

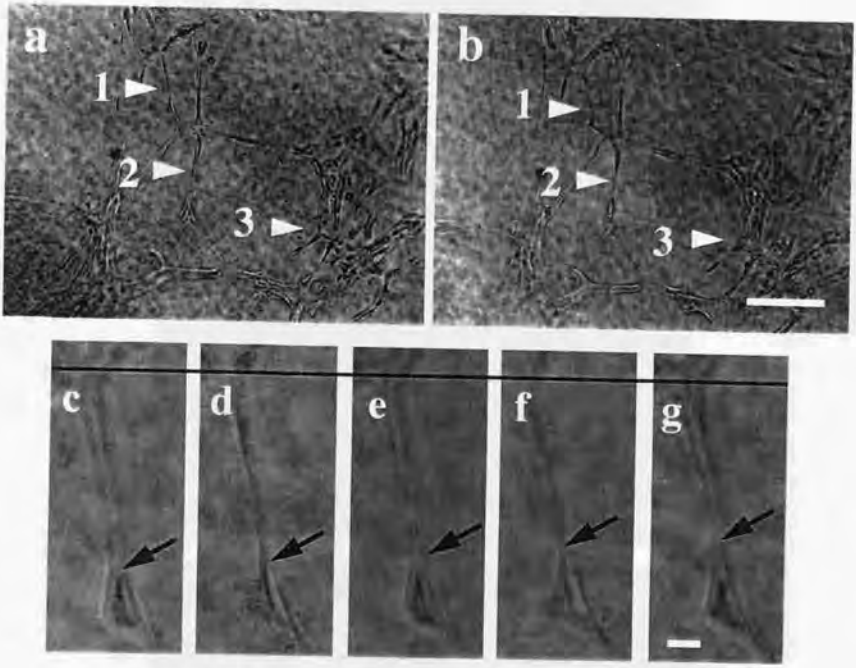


Fig. III-5. The effect of endothelin-1 on the contraction of M-SMC. M-SMC with 13 PDL cultured on rigid type IV collagen gel were treated with 1 nM endothelin-1 at culture day 3. The cells before (a) and after (b) addition of 1 nM endothelin-1 for 15 min. Arrowheads indicate representative points noted as having been moved upon addition of endothelin-1. The cell-body bent to be bow-like shape (arrowhead 1) and extended to be thin (arrowhead 2). Arrowhead 3 indicates that the curve made by two cells appeared to be smoothened. Bar; 100μm. Pictures with a higher magnification of the point indicated by the arrowheads 1 in (a) and (b) are shown in (c)-(g). Photographs were taken before (c) and after addition of 1 nM endothelin-1 at 1 min (d), 4 min (e), 10 min (f) and at 15 min (g). Apparently the same region of the cell body pointed by arrows moved upward. The black line being taken as the reference, the contracted distance was about 15 μm at 15 min. White bar; 10 μm.

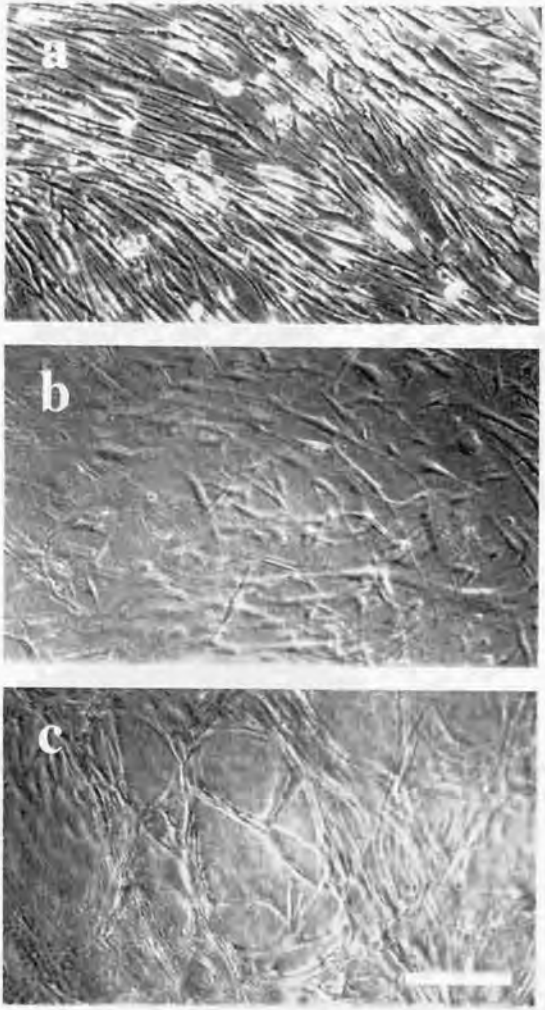


Fig. III-6. The effect of substrates on the morphology of cultured HF-18. HF-18 were cultured on type IV collagen-coated dish (a), type I collagen gel (b) or type IV collagen gel (c) in DMEM supplemented with 10% FBS. At culture day 5, the morphology of HF-18 was observed with the phase-contrast microscope and photomicrographed.
Bar; 100μm.

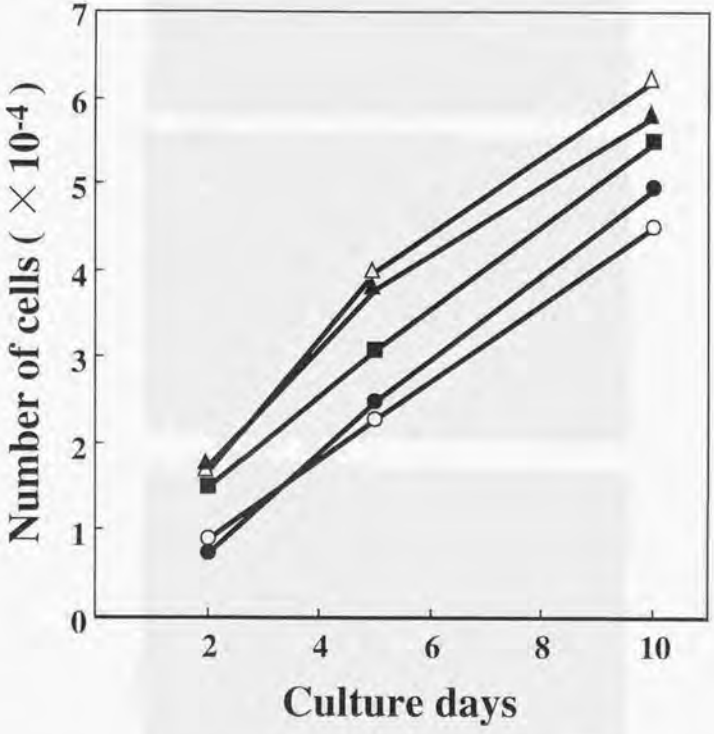


Fig. III-7. Growth curves of HF-18 cultured on different substrates. HF-18, normal human dermal fibroblasts, were cultured on plastic dish (■), type I collagen-coated dish (△), type IV collagen-coated dish (▲), type I collagen gel (○) or type IV collagen gel (●) in DMEM supplemented with 10% FBS for 10 days. The cells were initially plated at a cell density of 7.2×10^{3}/ well on 24 well culture dish. The medium was renewed every 3 days. The number of cells was counted on day 2, 5 and 10 with the Coulter counter after the cells were removed from the dish. Data represent the mean of three determinations in three separate experiments.

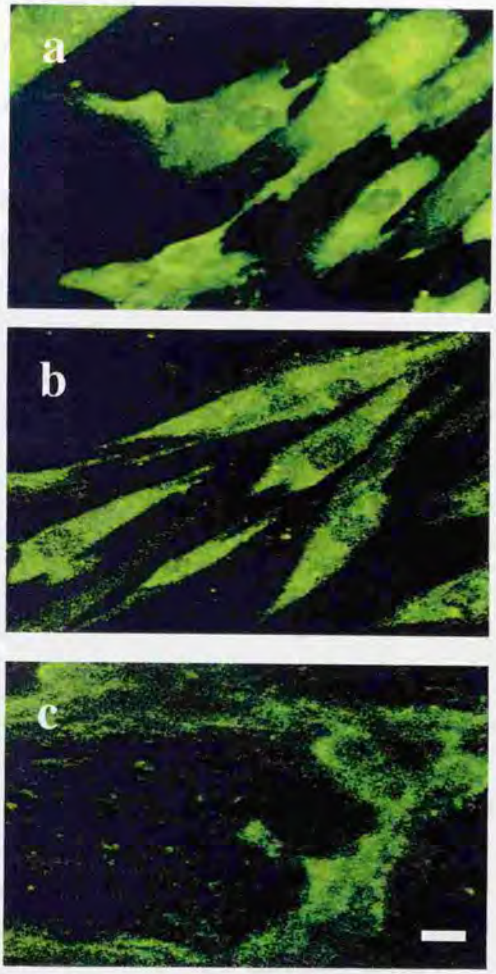


Fig. III-8. β1 integrin staining of M-SMC cultured on different substrates. M-SMC obtained from A-SMC through 5 times passages up to 13 PDL were cultured on type IV collagen-coated dish (a), type I collagen gel (b) or type IV collagen gel (c). At culture day 3, the cells were fixed and permeabilized at 25°C. Then they were incubated with anti-human β1 integrin monoclonal antibody at 4°C for 18 h. After exposure to FITC-prelabeled goat anti-mouse IgG, the cells were observed with the confocal laser scanning microscope and photomicrographed. No specific staining was observed in control cells (data not shown). Bar; 10μm.

General Discussion

My interest on the biological effects of ECM is focused on collagenous proteins, particularly comparison of the effects of molecules and supramolecular aggregates. The matrix architecture is in principle constructed through specific interactions of ECM components secreted from the cells as well as self-assembly. Furthermore, reiterative interactions between collagen aggregates and mesenchymal cells may modify arrangements of supramolecular aggregates. It has been reported that morphology and growth of fibroblasts much depended on the state of type I collagen, whether they are used as monomeric molecules or fibrillar gel (Nishiyama et al., 1989; Mercier et al., 1996), suggesting that strong interactions between the cells and consolidated extracellular collagen aggregates at least in the case of type I collagen and fibroblasts. Based on the findings, I extended the comparison of molecules with aggregates to the type IV collagen, since reconstituted type IV collagen gel consisting of polygonal meshwork has been available for cell culture substrates.

In the present thesis, I focused on the effects of the type IV collagen gel on the fundamental cellular behaviors such as growth and morphology of hepatic stellate cells and aortic smooth muscle cells including myofibroblasts as cell culture substrates. On the rigid type IV collagen gel, HSC and M-SMC showed a unique morphology with extremely elongated shape, eventually forming cell-to-cell junctions and stopped proliferation. Furthermore, growth suppression and induction of cell-to-cell junction on the rigid type IV collagen gel were also found for myofibroblasts that had acquired proliferating activities through many passages even in the presence of serum. The results suggest that the type IV collagen gel with sufficient rigidity may be a useful substrate for obtaining the differentiated HSC and A-SMC even from the cells with a synthetic phenotype. The results also indicate that extracellular environments, if appropriately arranged, could theoretically restore the myofibroblasts to the contractile cells. The rigid type IV collagen gel may become another candidate for a therapy of tissue fibrosis or

cirrhosis essentially distinct from the therapies which have been postulated.

What characteristics of the rigid type IV collagen gel is responsible for the specific effect on the cells? The gel rigidity or elasticity may in part depend on cross-bridges formed between NC1 domains and triple-helical domains of the type IV collagen (Nakazato et al., manuscript in preparation). Previous reports described the rigidity of type IV collagen gel depended greatly on the conditions and length of incubation time for the gel formation (Nakazato et al., 1996. Fig. 2; p. 12, 13). As far as I examined, formation of the rigid type IV collagen gel required a high concentration of the type IV collagen solution (more than 1.8 mg /ml) and long incubation time at 4°C (more than 5 days). The gel formed under the conditions reproduced the experimental phenomena such as, at least, formation of cell-to-cell junctions and growth suppression of the smooth muscle-related cells.

When the cells were placed on fragile gel, most of the cells came down to the plastic dish surface. Under these conditions, the cells behaved as they were placed on the non-gel form of type IV collagen aggregates (Fig. III-2c, III-2d). That is, the morphology of the cells looked spindle-like, typical for fibroblasts. Type IV collagen is distributed only at specific sites of normal liver sinusoids. However, in fibrotic or cirrhotic liver tissues, the distribution of the type IV collagen is scattered all over the tissues (Yoshida et al., 1997) The results tempted me to speculate that the disorganized supramolecular state of type IV collagen gel may cause uncontrolled proliferation and fibroblast-like cell shape of smooth muscle-related cells.

How far is the effect of the rigid type IV collagen gel extended to other differentiated cells? The rigid type IV collagen gel showed similar effects on myofibroblast-like kidney glomerular mesangial cells (M-MC) as found for HSC, M-HSC and M-SMC. That is, the rigid type IV collagen gel repressed the growth (Fig. 6) and induced the cellular morphologies to form eventually multicellular meshwork (Fig. 7). M-MC showed indistinguishable behaviors from HSC, M-HSC and M-SMC. Since the effective method

for repression of the cell proliferation has not been discovered, the rigid type IV collagen gel might provide a novel possibility to repress the growth of the cells. Another common feature may include the phenotypic transformation into fibroblast-like cells which produce fibrillar collagen as major secretory proteins and contract collagen gel when they are cultured within the reconstituted type I collagen gel. The collagen gel contraction is one of the characteristic functions of fibroblasts (Bell et al., 1979; Yamato et al., 1995; Lin et al., 1997). Thus, synthetic HSC, A-SMC and G-MC share many functional and morphological analogies with fibroblasts (Ehrlich et al., 1986; Desmouliére et al., 1995). They may sometimes be called myofibroblasts that may contribute to pathogenesis of fibrosis or sclerosis in the fibrotic or cirrhotic regions (Kent et al., 1976; Geerts et al., 1989). The morphology and proliferation in the response to type I collagen gel appear to be shared with HSC, A-SMC, G-MC, the myofibroblast-like cells and fibroblasts.

In vivo tissues, these cells are located in the intermediate between epithelial or endothelial tissues and connective tissues and have the contractile activities. Thus, they are classified as homologous cells distinct from endothelial cells, epithelial cells and fibroblasts. Preliminary examination revealed that epithelial cells (MDCK) and endothelial cells (A-EC) cultured on the rigid type IV collagen gel showed the essentially distinct behaviors from the smooth muscle-related cells; Spherical morphology was seen in spite of strong adhesion to the rigid type IV collagen gel (Fig. 8). These results imply that the rigid type IV collagen gel provides an appropriate extracellular signals to the cells, depending on the differentiation state *in vivo* tissues.

To my surprise, human rhabdomyosarcoma cells (RD) derived from skeletal muscle cells showed quite a similar appearance to the smooth muscle-related cells; The cells also formed cellular junctions and showed little proliferative activity on the rigid type IV collagen gel, while RD extremely elongated and proliferate actively at an initial on the collagen-coated dish and the type I collagen gel (Fig. 9, Fig. 10). Tumor cells including RD were surrounded by and close contact with type IV collagen, suggesting that finely

organized structure of type IV collagen like the rigid type IV collagen gel may required in the growth-suppression of tumor cells.

Appropriate interactions between differentiated cells and ECM environments, collagen aggregates in particular, might be play important roles in the formation of tissue and organ structure. The multicellular system composed of the finely organized type IV collagen supramolecular aggregates in a gel form together with the mesenchymal cells including smooth muscle-related cells might be a useful system to evaluate the tissue functions *in vitro* and could be a promising biomaterial for generation of functional organs *in vivo*.

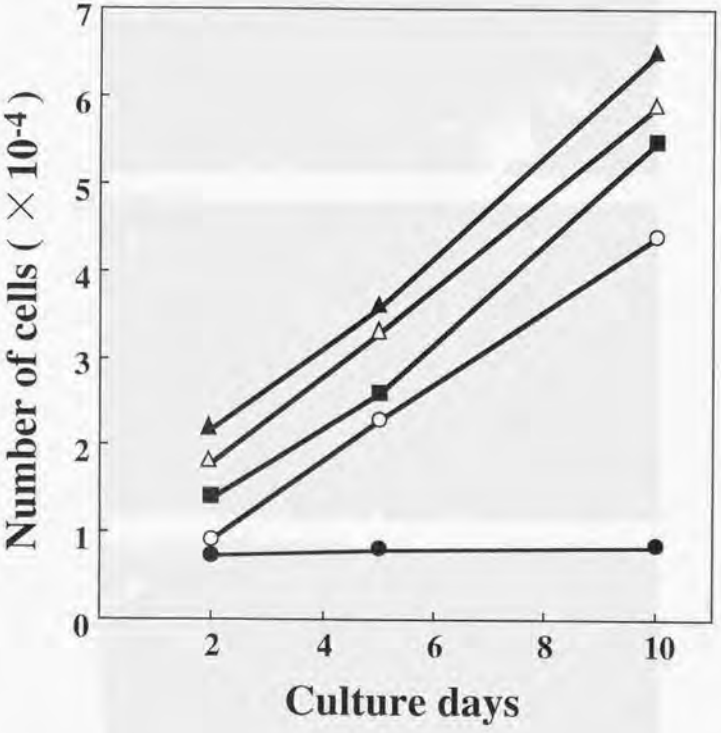


Fig. 6. Growth curves of myofibroblast-like glomerular mesangial cells. M-MC were cultured on plastic dish (■), type IV collagen-coated dish (▲), type I collagen gel (○) or type IV collagen gel (●) in MCDB 131 medium supplemented with 10% FBS for 10 days. The cells were initially plated at a cell density of 7.2×10^3/ well on 24 well culture dish. The medium was renewed every 3 days. The number of cells was counted on day 2, 5 and 10 with the Coulter counter after the cells were removed from the dish. Data represent the mean of three determinations in three separate experiments.

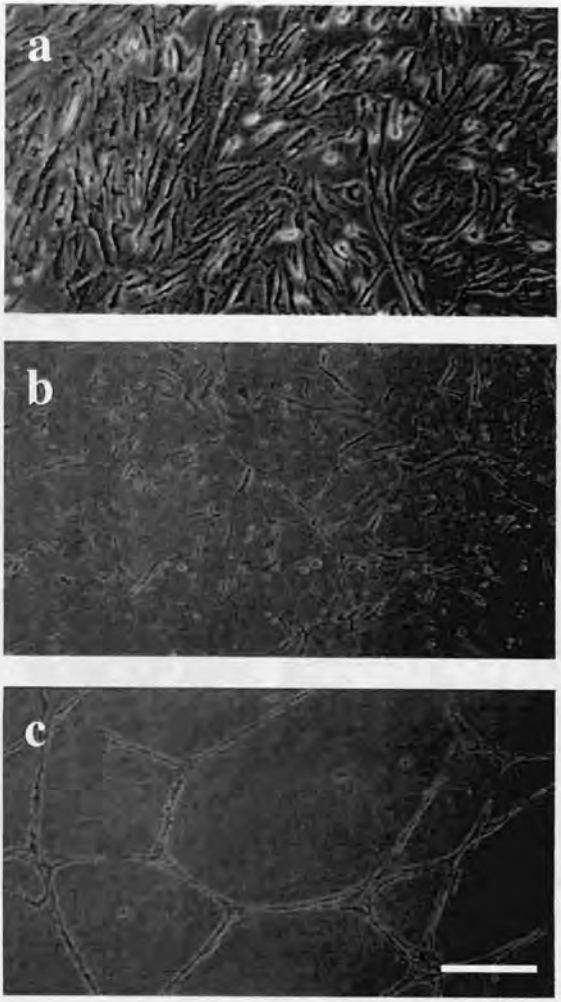


Fig. 7. Morphology of myofibroblast-like glomerular mesangial cells. M-MC were cultured on type IV collagen-coated dish (a), type I collagen gel (b) or type IV collagen gel (c) in MCDB 131 medium supplemented with 10% FBS. At culture day 5, the morphology of M-MC was observed with the phase-contrast microscope and photomicrographed. Bar; 100μm.

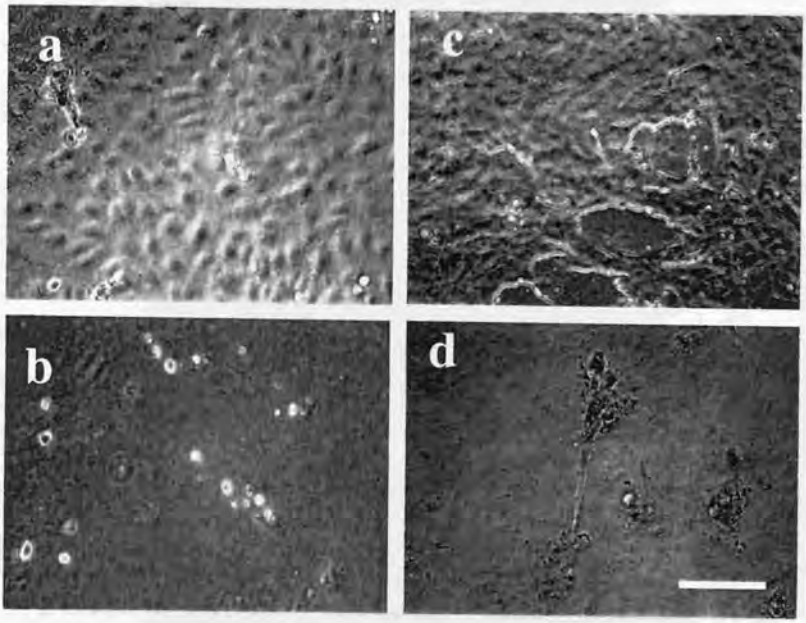


Fig. 8. Morphology of human aortic endothelial cells and MDCK cells. A-EC and MDCK cells were cultured on type I collagen gel (a, c) or type IV collagen gel (b, d). A-EC (a, b) were grown in the medium as described in Experimental Procedures. MDCK cells (c, d) were cultured in DMEM containing 10% FBS. At culture day 5, the morphology of A-EC and MDCK cells were observed with the phase-contrast microscope and photomicrographed. Bar; 100μm.

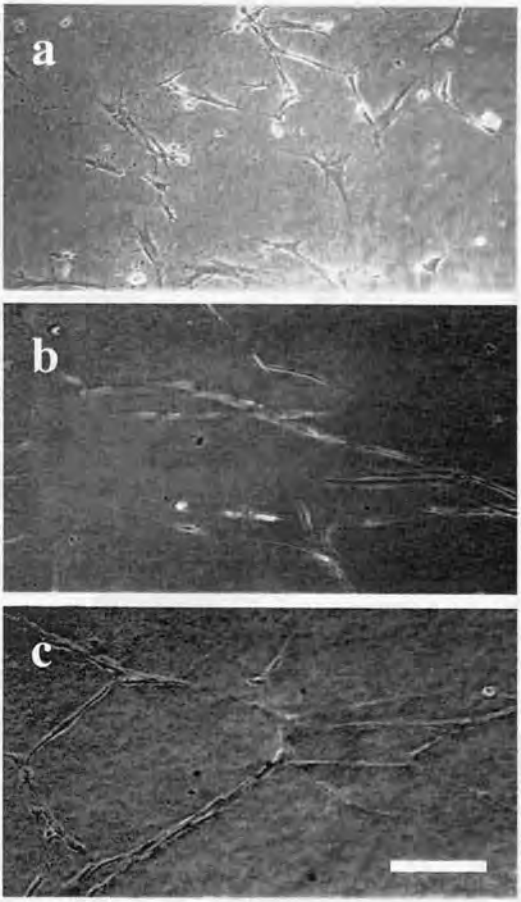


Fig. 9. Morphology of human rhabdomyosarcoma cells. RD cells were cultured on type IV collagen-coated dish (a), type I collagen gel (b) or type IV collagen gel (c) in DMEM supplemented with 10% FBS. At culture day 5, the morphology of RD was observed with the phase-contrast microscope and photomicrographed. Bar; 100μm.

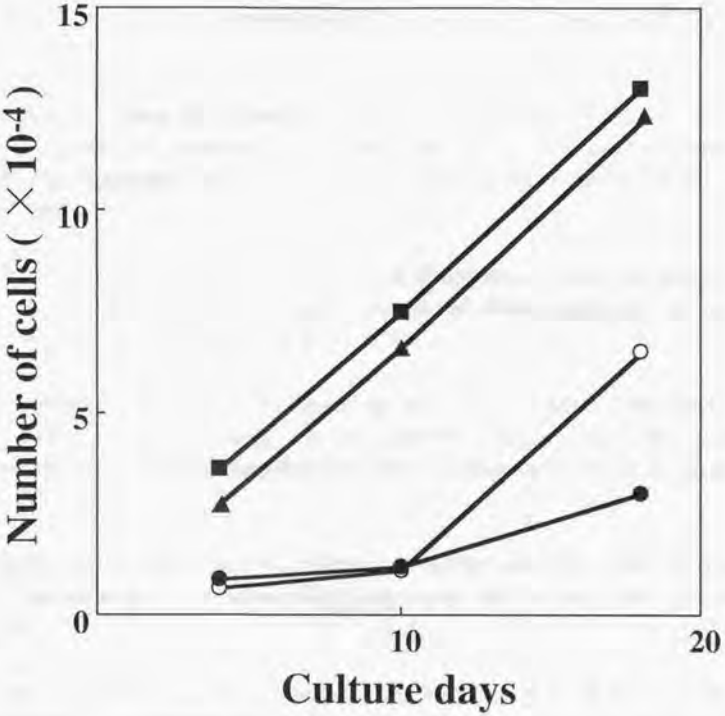


Fig. 10. Growth curves of human rhabdomyosarcoma cells. RD cells were cultured on plastic dish (■), type IV collagen-coated dish (▲), type I collagen gel (○) or type IV collagen gel (●) in DMEM supplemented with 10% FBS for 18 days. The cells were initially plated at a cell density of 7.2×10^3/ well on 24 well culture dish. The medium was renewed every 3 days. The number of cells was counted on day 4, 10 and 18 with the Coulter counter after the cells were removed from the dish. Data represent the mean number of the cells of three determinations in three separate experiments.

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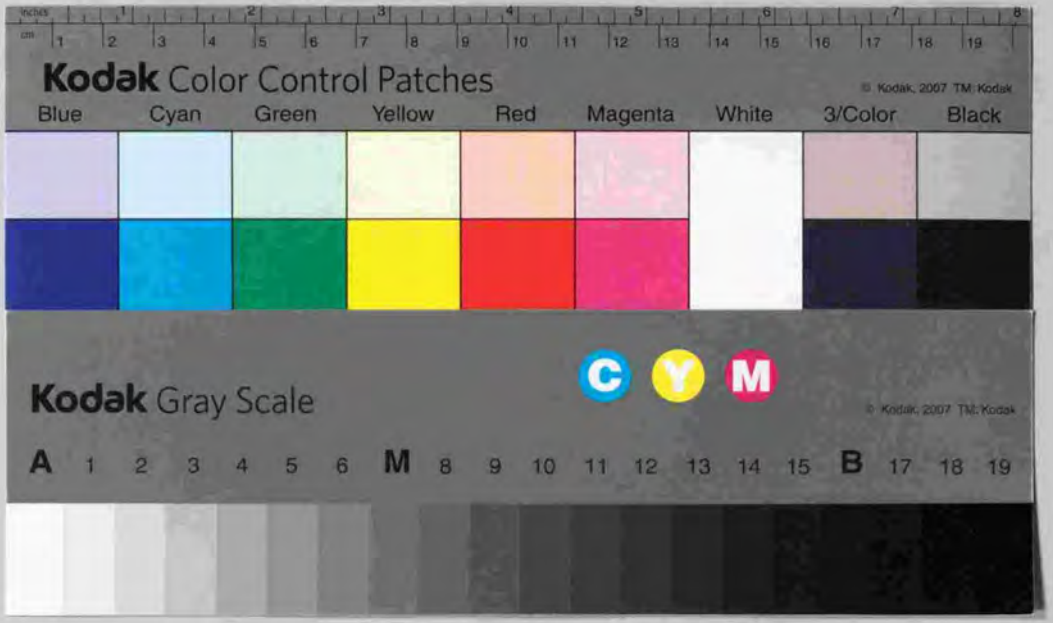
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