Characteristics of the Human Dermal Fibroblasts Cultured within Reconstituted Collagen Fibrils

再構成コラーゲン線維ゲル内培養におけるヒト皮膚線維芽細胞の特徴

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Characteristics of the human dermal fibroblasts cultured within reconstituted collagen fibrils

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Contents

(Pages)

General Introduction (1)

Chapter I. Collagen gel contraction madiated by human dermal fibroblasts. (7)

Summary (7)
1. Introduction (8)
2. Materials and Methods (9)
3. Results (12)
3.1 Effect of culture condition (12)
3.2 Effect of collagen concentration (13)
3.3 Effect of donor ages or population doubling levels of human fibroblasts (13)
3.4 Effect of drugs which act the state of cytoskeleton (14)
3.5 Relationship between the length of lag phase of collagen gel contraction and the population of elongated fibroblasts (15)
4. Discussion (16)
Tables and Figures (22)

Chapter II. Morphology of human dermal fibroblasts in three-dimensional collagen gel (31)

Summary (31)
1. Introduction (32)
2. Materials and Methods (33)
3. Results (36)
3.1 Surface structure of fibroblasts cultured in a collagen gel (36)
3.2 Organization of actin microfilaments in fibroblasts cultured in a collagen gel (37)
3.3 Morphological change of fibroblasts

in a collagen gel (38)
3.4 Organization of fibroblast cytoskeleton in a collagen gel after treatment with the low dose of cytochalasin D (39)
4. Discussion (39)
Figures (44)

Chapter III.Growth rate of human dermal fibroblasts cultured within three-dimensional collagen gel (52)

Summary (52)
1. Introduction (53)
2. Materials and Methods (55)
3. Results (59)
3.1 Comparison of cell proliferation by "in-gel" culture with "on-gel" culture (59)
3.2 Diffusion of nutrients from culture medium into collagen gel (60)
3.3 Relation between reduction in growth rate and contarcted gel size (61)
3.4 Density of collagen fibrils in contracted collagen gel (62)
4. Discussion (62)
Tables and Figuers (68)

Chapter IV. Characteristic of the quiescent state of human dermal fibroblasts in contracred collagen gel. (73)

Part A : No response to epidermal growth factor and its stimulant (74)

Summary (74)
1. Introduction (75)
2. Materials and Methods (76)
3. Results (80)
3.1 Effect of reconstituted type I collagen gel

on growth response of fibroblasts to EGF (80)
3.2 Growth response to EGF of quiescent fibroblasts in the contracted collagen gel (81)
3.3 Binding of EGF to quiescent fibroblasts in the contracted collagen gel (82)
3.4 Mitotic effect of a combination of EGF and saikosaponin b_1 on the growth of quiescent fibroblasts in the contracted collagen gel (82)
4. Discussion (83)
Tables and Figures (88)

Part B : Growth response to other growth factors (96)

Summary (96)
1. Introduction (97)
2. Materials and Methods (98)
3. Results (100)
3.1 Growth response of quiescent fibroblasts on plastic dishes to growth factors (100)
3.2 Mitogenic effect of EGF, b-FGF, PDGF or TGF-β on human dermal fibroblasts growth-arrested in the contracted collagen gel (101)
4. Discussion (102)
Tables and Figures (105)

General Discussion (110)

Conclusion and Perspective (114)

Acknowledgments (115)

References (116)

General Introduction

Extracellular matrix, in or upon which cells reside *in vivo* tissue, has been expected to play an important role in regulating morphology, migration, proliferation, biosynthesis, differentiation or other functions of cells. Followings are some examples which demonstrate the role of extracellular matrix in cellular functions *in vivo*. First, in the skin the stem cells of the epidermis lying in the basal layer in contact with a basement membrane retain the potential of proliferation (Green, 1977; Watt, 1984; Barrandon and Green, 1987). The progeny of the stem cells lose proliferating potential upon leaving this layer. Second, each muscle cell in the adult muscular tissues is enveloped in the basement membrane. When the muscle is damaged, it degenerates and dies, and macrophages move in to clear away the debris. If the basement membrane remained, it would provide a scaffolding within which new muscle fibres can be constructed from surviving stem cells (Burden et al., 1979; Alberts et al., eds., 1989-d). Third, if a preparation of bone matrix, made by grinding bone into a fine powder and dissolving away the hard mineral component, is implanted in the dermal layer of the skin, dermal fibroblasts become transformed into cartilage cells and, a little later, others into bone cells (Gabbiani and Rungger-Brandle, 1981; Alberts et al., eds. 1989-c). From these studies, the extracellular matrix is thought to be one of the major factors for regulating the cellular behavior in a given tissue.

Epithelial cells, whose basal surfaces are in contact with extracellular matrix, organize their cortical cytoplasm and remain on top of an extracellular matrix organization, called basement membrane whose structure is sheetlike multilayered meshwork composed by type IV collagen, laminin, heparan sulfate proteoglycans and so on (Sugrue and Hay, 1981; Kleinman et al., 1986; Watt, 1986; Yurchenco and Ruben, 1987; Timpl, 1989; Alberts et al., eds., 1989-b,c). On the other

hand, mesenchymal cells including fibroblasts tend to burrow into hydrated lattices of extracellular matrix which is mainly composed by the fibrous collagens (such as type I, II, III or V collagen) and once inside the lattices of collagen fibrils, they assume morphologies and functions that are appropriate for their particular tissue type (Bell et al., 1979; Coulomb et al., 1983; Schor et al., 1983; Tomasek and Hay, 1984; Nusgens et al., 1984; Colige et al., 1988; Alberts et al., eds., 1989- b,c).

The main components of extracellular matrix in connective tissue such as the dermis are the fibrous collagens (mainly type I, III and V). These were initially thought to be stable and thus relatively inactive constituent of connective tissue to the cells, while collagens are the most abundant proteins in the tissue. The mechanism of collagen fibril formation, the biochemical properties of collagens and the metabolism of collagens have been the main studies. Little interest was paid to the biological ativity of collagens in terms of influencing cellular behavior. Recently, it becomes increasingly obvious that collagens play important roles in regulating cellular activities including morphogenesis, development, cellular differentiation, etc. (Alberts, et al., eds., 1989-b; Akiyama et al., 1990). Collagens are recently known to be adhesion molecules that bind to cells as well as fibronectin which is well-known to be a adhesive glycoprotein in cell culture. However, the mechanism of effect of collagen on cellular events is little known as compared to that of fibronectin. Many putative collagen receptors have also been reported, but none have yet been characterized (Akiyama et al., 1990). It is presumed that the effects of collagen on cells might be mediated through specific cell surface receptors (Carter and Wayner; 1988). The effect of collagen on cellular behavior is limited and analysis of the interactions between collagen and cells has been lacking.

The implications of extracellular matrix function in vivo tissue are mainly provided from the studies on the cellular

behavior in culture. Distinct behavior of fibroblasts cultured with collagen substrata from that of the cells on plastic dishes is intriguing, in that the cellular environment is more similar to that in the tissue or organ than is the case in a flat plastic dish (Bell et al., 1979; Sarber et al., 1981, Nusgens et al., 1984; Saiag et al., 1985; Colige et al., 1988; Mauch et al., 1988). Reconstituted collagen fibrils have a packing arrangement of the individual collagen molecules: they are staggered, so that adjacent molecules are displaced longitudinally by almost one-quarter of their length (a distance of 67 nm). This arrangement presumably maximizes the tensile strength of the aggregate. The effect of the orderly alignment of interaction sites with cells can only be examined with collagen but not with other extracellular matrix components. Furthermore, in the culture system the ability to suspend fibroblasts within gelling solutions of collagen provides an opportunity to examine cell-matrix interactions in vitro that closely resemble the in vivo counterpart and to analyze the influence of collagen matrix on cellular behavior. It is another interesting point for the cell culture with collagen substrata that different states of interaction between fibroblasts and collagen can be provided by using the reconstituted matrix of collagen. For example, the cells can be cultured either on top of or inside of three-dimensional collagen matrix. The method will bring an information of the distribution and density of interaction sites between a cell and collagen under different conditions.

The effects of collagen on fibroblast behavior, particularly cell morphology and cell growth, are summarized as follows. In regard to cell growth, it has been reported that normal fibroblasts proliferated more rapidly on plastic dishes than on the surface of collagen gel or within collagen gel (Schor, 1980; Schor et al., 1982; Buttle and Ehrlich, 1983). Elsdale and Bard (1972) reported that cells cultured on collagen substrata showed reduction in ^{3}H-thymidine incorporation. Yoshizato et al. (1984 and 1985) have shown

that ^{3}H-thymidine incorporation was suppressed by the cells which were cultured on native collagen-coated dishes, but not by the cells cultured on denatured collagen-coated dishes. Sarber et al. (1981) showed that human fibroblasts in contracting collagen gel ceased to incorporate ^{3}H-thymidine into DNA after 4 days of culture. On the contrary, some previous reports indicated that collagen matrix stimulates the growth of fibroblasts by the collagen accumulation on the cell surface (Hata and Senoo, 1989) or fibroblasts cultured in collagen gel showed increase in ^{3}H-thymidine incorporation (Nakagawa et al., 1989). The previous reports are classified into two opposite results. One group claims that native collagen represses fibroblast proliferation and the other is that collagen stimulates the growth. The apparent contradiction has not been resolved.

Cellular behavior of the culture within reconstituted collagen gel ("in-gel" culture) has not been quantitatively distinguished from that of the culture on the surface of collagen gel ("on-gel" culture). In most cases, the presence of collagen in native forms has been compared with the absence or presence of denatured forms of collagen. Fibroblasts *in vivo* tissue usually reside in extracellular matrix which is mainly composed of collagen fibrils. The "in-gel" culture system thus should at least give topologically similar situation to the cells. It has also been reported that cell proliferation was further more strongly repressed in contracted collagen gel by fibroblasts than "in-gel" culture without contraction (Sarber, et al., 1981; Nakagawa et al., 1989). The state of fibroblasts in contracted gel might be similar to that *in vivo* tissue in terms of density of the collagen fibrils surrounding cells. However, characteristics of the quiescent state of fibroblasts cultured in contracted collagen gel are yet to be analyzed.

In regard to cellular morphology, it has been reported that fibroblasts cultured within the reconstituted collagen gel ("in-gel" culture) became extremely elongate in shape as

compared to the spindle-like shape on plastic dish (Buttle and Ehrlich, 1983; Tomasek and Hay, 1984; Delvoye et al., 1986). The elongation process is induced by the interaction of cells with collagen fibrils. Cytoskeletal organization may well contribute to the process. Tomasek and Hay (1984) have reported the role of cytoskeleton in the collagen-induced morphological change of embryonic chick corneal fibroblasts within collagen gel. It was suggested that both actin microfilaments and microtubules were required for elongation process of corneal fibroblasts within collagen gel. However, the difference between "in-gel" culture and "on-gel" culture was not examined. Cytoskeletal organization is known to be closely coupled with cell adhesion (Burridge et al., 1988). Therefore, distribution of collagen attachment to cell surface might be important for morphology and cytoskeletal organization of fibroblasts. The distinctive treatment of "in-gel" culture from "on-gel" culture may also be crucial in the effect on cellular shape and its change of fibroblasts.

The present thesis is focused on the effect of reconstituted collagen matrix in three-dimensional surroundings to cells on cellular behavior of human dermal fibroblasts including cell morphology, cytoskeletal organization, cell proliferation and growth responsiveness to peptide growth factors. In Chapter I, collagen gel contraction by human dermal fibroblasts has been attempted to analyze phenomena quantitatively. Various factors affecting the gel contraction governed the contraction process. Phenomenologically, cell morphology or cytoskeletal organization was found to be correlated with the timing of initiation of gel contraction. Observations in Chapter II included that morphology and cytoskeletal organization of fibroblasts within collagen gel are distinct from those on the gel and that three-dimensional surroundings of collagen fibrils retarded morphological change of fibroblasts from round shape and that low dose of cytochalasin D reversed the retarding effect of collagen, indicating that actin

microfilaments repressed the elongation of fibroblasts in collagen gel rather than contributed to elongation process as might have been expected. The effect of three-dimensional collagen gel on cell growth has been analyzed in Chapter III and IV. The growth rate and the growth response to growth factors were repressed when fibroblasts were cultured within collagen gel, but not on the gel. Fibroblasts in contracted collagen gel containing high density of collagen fibrils stopped multiplying, even in the culture with 10% fetal bovine serum. The growth repression by collagen fibrils in collagen gel was not due to direct cell-cell contact by histological examination. Cell density was far less than confluency (Chapter III). Distinctness of the quiescent state in contracted collagen gel, which we call cell-matrix contact inhibition, was obtained in the response of the cells to growth factors in Chapter IV. Sevral growth factors did not stimulate the proliferation of fibroblasts which are growth-arrested in contracted collagen gel, while platelet-derived growth factor showed a marked stimulation of cell growth. From the results, collagen fibrils in contracted gel might affect mitogenic signaling pathways in the cells presumably through integrins and cytoskeletons. The distribution and/or density of collagen-cell interaction sites are proposed to be one of the crucial factors regulating the cellular behavior of human dermal fibroblasts.

Chapter I

Collagen Gel Contraction Mediated by Human Dermal Fibroblasts

[Summary]

Kinetics of collagen gel contraction by fibroblasts cultured in vitro was examined in detail for quantitative analysis. The process of collagen gel contraction was not expressed by a simple function of time. It appeared to consist of three distinct phases ; a lag phase before the initiation of contraction, a rapid contraction phase and a slow contraction phase. Factors affecting the gel contraction can be classified into four groups. The first group includes increase in cell number, in culture temperature or in serum concentration, which strengthened the contraction in all the three phases, suggesting that they affected cellular activity paticularly in interacting with collagen. The second group repressed the later two phases of contraction but not the first lag phase, typically increase in collagen concentration and a low dose of nocodazole or colcemid. Increasing population doubling levels of fibroblasts belongs to the third group which caused a reduced lag time but no change in the later two phases. Cytochalasin D at a low dose (0.03-0.1 μg/ml) is another example of the third group which shortened the lag time. The last group did not change the contraction curves. Donor age of fibroblasts isolated from the skin is an example of this group. The rate of rapid contraction in the second phase was always found to be closely correlated with the degree of contraction at the end of the third phase, in a whole set of the factors above mentioned. The results suggest that the extent of the later two phases might be a reflection of the same cellular activity, particularly cytokinetical one. The lag time is directly related to the time for cells to

become elongate in shape as observed by using the video-microscopy, suggesting that the lag phase is also governed by cytokinetical activity. We conclude that collagen gel contraction is essentially separated into two diferent phases, a lag phase and a contracting phase, and each phase is governed by two different cytokinetical activities due to actin filament dynamics.

1. Introduction

The morphology and function of fibroblasts are influenced by the extracellular matrix (Tomasek et al., 1982; Kleinman et al., 1981). Recently, the culture of fibroblasts in a collagen gel has been adopted for studying cellular behaviors which approximate cell-matrix interactions in vivo (Bell et al., 1979; Nusgens et al., 1984; Guidry and Grinnell; 1986). Fibroblasts incorporated in a collagen gel attach rapidly to collagen fibres, become elongate in shape (Tomasek and Hay, 1984), and then contract the collagen gel. These fibroblast-collagen interactions result in rearrangement and increased density of the collagen fibrils (Bell et al., 1979; Guidry and Grinnell, 1985). The ability of fibroblasts to contract collagen gels is one of fibroblast specific functions which might be closely coupled with cell attachment to collagen fibrils and the cytoskeletal organizations.

Factors affecting the fibroblast-mediated collagen gel contraction, such as the number of cells present, the collagen concentration, the nature of cells in question, and drugs which modified the cellular activity have been described in the previous reports (Bell et al., 1979; Steinberg et al., 1980; Ehrlich et al., 1983; Ehrlich and Wyler, 1983; Buttle and Ehrlich, 1983; Van Bockxmeer et al., 1984; Delvoye et al., 1986; Adams and Priestley, 1986; Gillery et al., 1986; Ehrlich et al., 1986(a) and 1986(b)). Most of the previous works on

collagen gel contraction treated the process as a simple step and thus the data are semi-quantitative and too much complicated to be compared quantitatively among different experiments which were performed under distinguished conditions.

The present study on the collagen gel contraction phenomena has been directing toward a more quantitative analysis to obtain information of the fibroblast-collagen interaction in detail. We have demonstrated by examining the process in detail that it involved three distinct phases in the time course curve; a lag phase before the initiation of contraction, a phase of rapid contraction, and a phase of slow contraction (terminal phase). The parameters representing the three phases are lag time (h), rate of contraction (%/h), and percentage of gel size contracted on day 6, respectively. From the present analysis, we have obtained some new findings as to the action of cytochalasin D and the effect of population doubling level of normal human fibroblasts on collagen gel contraction.

2. Materials and Methods

Cell culture

Human foreskin fibroblasts from a 0-year-old male (HF-0) and human fetal fibroblasts from 4-month fetal skin and 5-month fetal skin (HEF-4 and HEF-5) were a kind gift from Dr. Kawamoto, Department of Biochemistry, Okayama University Dental School (Sato et al., 1983). Human skin fibroblasts from a 1-year-old male (HF-1) were also a kind gift from Dr. Shinkai, Department of Dermatology, Ohita Medical School. Other dermal fibroblasts were isolated from human skin obtained during plastic surgery from a 30-year-old female (HF-30), a 41-year-old male (HF-41), a 60-year-old female (HF-60), a 69-year-old female (HF-69), a 71-year-old female

(HF-71). Briefly, the cultures were initiated by cell outgrowth from explants of the dermis. The primary cultures were grown in Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical Co., Tokyo) supplemented with 10% fetal bovine serum (FBS, Gibco Labs., New York), 3.7 g/l sodium bicarbonate, 50 IU/ml penicillin (Banyu Pharmaceutical Co., Tokyo) and 50 µg/ml streptomycin (Meiji Seika Kaisha, Tokyo). Subconfluent cells were dispersed with 0.1% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS, pH 7.4) and propagated in the above culture medium. Cell cultures were maintained in 25 cm^2 flasks and passaged at a 1:4 split ratio when the cultures reached confluency. The number of population doublings per passage were estimated by the following equation: $x=(\log N - \log N_0)/\log 2$ where x= number of population doublings, N_0= number of cells initially plated and N= number of cells present in confluent cultures before passage. Under the standard conditions using a split ratio of 1:4, a mean population doubling of 2 per passage was obtained. Cells at indicated population doubling level (PDL) were frozen and kept in liquid nitrogen.

Preparation of collagen gels containing fibroblasts

The cells just before reaching confluency were removed from the dishes by trypsin treatment (0.1% trypsin and 0.02% EDTA in PBS for 10 min at 37°C), washed with the culture medium, counted and suspended to an appropriate concentration. A solution of pepsin-treated type I collagen (porcine tendon) at a concentration of 3 mg/ml was obtained from Nitta Gelatin Co. (Osaka). The collagen gels were prepared by mixing 1 ml of 3x concentrated DMEM with sodium bicarbonate, penicillin and streptomycin, 0.33 ml of FBS, 2 ml of the collagen solution and 6.67 ml of DMEM containing 10% FBS at 4°C. Two ml of the cell suspension in DMEM containing 10% FBS (6.0×10^4 cells/ml) was added and mixed with the medium at 4°C to give a final density of 1.0×10^4 cells/ml and a collagen concentration of 0.5 mg/ml. Two ml of the medium containing

cells and collagen was placed in a bacteriological plastic dish, 32 mm in diameter, and then brought to 37°C for allowing polymerization of the collagen. The incubation was continued at 37°C under an atmosphere of 95% air and 5% CO_2. Under the conditions, the collagen polymerized rapidly so that the cells were trapped in a three-dimensional collagen matrix. Collagen gels containing cells were detached from periphery of plastic dishes by scraping on passing 3 h after starting the gel culture. This is a requisite for maximizing reproducibility of the experiments, particularly the measurement of lag time. The culture medium was renewed every 2 days. The diameter of gels was measured at the indicated time.

Video recording of fibroblasts within collagen gels

Two ml of the medium containing cells and collagen which was prepared under the same condition of the above experiment was placed in a tissue culture dish and then brought to 37°C. Fibroblasts cultured within the collagen gel were observed with a phase-contrast microscope or a differential interference contrast microscope (Nikon, Tokyo). The stage was surrounded by a plastic chamber and kept under an atmosphere of 95% air and 5% CO_2 and at 37°C by applying heated air intermittently. Magnified views of fibroblasts in collagen gels were recorded on video tape for 0.1 second at intervals of 30 seconds for 3 days by using a video-recording system (Sankei Co., Tokyo).

Other Materials

Cytochalasin D, nocodazole and colcemid were purchased from Aldrich Chemical Co. (Milwaukee), Janssen (Belgium) and Wako Pure Chemical Industries (Osaka), respectively.

3. Results

3.1 Effects of culture conditions

The contraction of collagen gel by human foreskin fibroblasts (HF-0, 15th PDL) in terms of gel size was affected by changing culture conditions ; cell number, FBS concentration and culture temperature. Time course curves of contraction in detail with various cell number were shown in Fig. I-1. All the curves appeared to consist of three distinct phases ; the first phase is a lag time before the initiation of contraction, the subsequent phase is a rapid contraction of gel and the third phase is a very slow contraction with a slight decrease in diameter of the gel. Three phases in the time course curve were discernible under different FBS concentrations or culture temperatures (data not shown). Each phase can be quantitatively represented by parameter defined as follows. The first phase is expressed by length of lag time. Rapid contraction in the second phase showed a straight line over a nearly whole range and thus the rate of contraction was obtained by slope of time course curve in % decrease in gel diameter per unit time. In the third phase, the extent of contraction was expressed by the percentage contracted on day 6 [(Initial gel size in diameter - gel size in diameter on day 6) x 100/Initial gel size in diameter], when contraction is extremely slow, reaching bottom.

Effects of cell number, FBS concentration and culture temperature on the three phases of contraction were evaluated by these parameters in Fig. I-2 and I-3. Reducing the cell number decreased the efficiency of contraction in all three phases. Varying the concentration of FBS in the collagen gel up to 10% also affected all three phases. At 10% of FBS, the parameters reached plateau. Culture temperature also influenced all three phases. The relationship among the three distinct phases in the time course curves is shown in Fig. I-2

and I-3. Variations in cell number, FBS concentration or culture temperature give rise to values of the lag time which appear to be closely correlated to the rate of contraction in the second phase in case of both HF-0 and HF-30 (Fig. I-2). Relation between the second phase and the third phase is approximately expressed by straight lines which follow an equation ; Rate of contraction (%/h) = [100 x A]/[% of gel size contracted on day 6] + C ,(A and C are adjustable constants). The whole set of data obtained in the present study are on either one of the straight lines (Fig. I-3). HF-0 showed a prolonged lag time and a decreased rate of contraction as compared with those of HF-30.

3.2 Effect of collagen concentration

Influence of collagen concentration in the three phases of contraction is shown in Table I-1. The rate of contraction and the percentage contracted were repressed by increasing the initial collagen concentration in the gel. On the contrary, the length of lag time was not affected by the initial concentration of collagen at the range examined.

3.3 Effect of donor ages or population doubling levels of human fibroblasts

Normal fibroblasts with different proliferative potential were isolated from human skin of different donor ages. Collagen gel contraction was measured by using the cells of 8th to 12th PDL. Kinetic parameters in three phases of contraction were identical among the fibroblasts examined (Table I-2). Collagen gel contraction was apparently independent of donor age of fibroblasts. The effects of PDL of human fibroblasts on collagen gel contraction were shown in Fig. I-4 and in Table I-3. The in vitro senescent cells had as strong an ability to contract the gels as the young cells. The high PDL cells shortened the lag phase. Human fibroblasts,

particulary foreskin fibroblasts, revealed the reduction of lag time with increasing PDL. However, the subsequent two phases which were indicated by the contraction rate and the percentage contracted were identical between low PDL and high PDL. Fibroblasts of the highest PDL (foreskin fibroblasts of 46th PDL and skin fibroblasts of 28th PDL) are approaching *in vitro* senescence in that the cells abolished the proliferation.

3.4 Effect of drugs which act on the state of cytoskeletons

The contraction of collagen gel by human foreskin fibroblasts (HF-0, 15th PDL) was investigated at drug concentrations of 0.001 μg/ml to 1.0 μg/ml. Low doses of nocodazole or colcemid which is known to disrupt microtubules repressed the contraction rate and the percentage contracted, but the lag time was not affected by the drugs (Table I-4). Collagen gel contraction was completely inhibited by the drugs at concentrations of more than 0.1 μg/ml. The effects of cytochalasin D which is known to act primarily on the state of actin are shown in Fig. I-5. Low doses of cytochalasin D (0.03 and 0.1 μg/ml) induced the reduction of lag time (32 h to 10 h), but repressed the contraction rate in the second phase. The low dose of cytochalasin D induced the elongation of fibroblasts from spherical cell shape incorporated in collagen gels and almost all cells have been already elongated in shape at 10 h after starting the gel culture (Fig. I-6). In the absence of cytochalasin D, most cells are in spherical shape at this stage. Cytochalasin D at concentrations of more than 0.3 μg/ml completely prevented contraction. Furthermore, upon addition of cytochalasin D, nocodazole or colcemid at a concentration of 0.1 μg/ml to the gel which was rapidly contracting in the second phase, contraction suddenly stopped (data not shown).

3.5 Relationship between the length of lag phase of collagen gel contraction and the population of elongated fibroblast

Morphological change of human foreskin fibroblasts of 15th PDL or 45th PDL which showed different length of lag time of collagen gel contraction (Fig. I-4) was observed by video microscopy. Features that were observed included the development of extremely elongated cell shape from spherical trypsinized cells and interaction between elongated pseudopodia and collagen fibrils. Mitotic figure of cells was not observed for 42 h after starting the gel culture.

A typical example of the morphological change of fibroblasts during the lag phase of collagen gel contraction is shown in Fig. I-7. Trypsinized fibroblasts initially remained spherical in collagen gel and the cells adhered to, compressed and stretched the collagen fibers. The cells began to extend pseudopodia and the length increased. Eventually the cells became bipolar and extremely elongated in shape. For classification criteria, cells which maintained the elongated shape with a length more than 150 μm were taken as "elongate". Fifty % of fibroblasts in the microscopic field became "elongate" in shape at about 28 h for the cells of 15th PDL and at about 11 h for the cells of 45th PDL. Fibroblasts of high PDL underwent morphological change from sphere to elongate in collagen gel for a shortened time. The period in which 50% of the cells became elongated in shape was also dependent on culture temperature ; 85 h at 32.5 °C and 28 h at 37.0 °C. The lag time before initiation of collagen gel contraction was strongly correlated to the time length for average fibroblasts to take elongated shape.

4. Discussion

Collagen gel containing fibroblasts in culture eventually contracts due to specific interactions between living cells and collagen fibrils. The contraction is not due to action of secreted factors by the cells such as proteolytic enzymes. When trypsinized fibroblasts are cultured in collagen gels, a characteristic morphological change of spherical fibroblasts to extremely elongated shape, distinct from a spindle-like shape on plastic dish, is observed (Tomasek et al., 1982; Tomasek and Hay, 1984). Concomitantly, the collagen gel initiates contraction when number of the elongated fibroblasts increased sufficiently (Bell et al., 1979; Buttle and Ehrlich, 1983; Ehrlich et al., 1983). Various factors affecting the collagen gel contraction have been exploited in previous reports for elucidation of contraction mechanism (Bell et al., 1979; Steinberg et al., 1980; Buttle and Ehrlich, 1983; Ehrlich et al., 1983; Adams and Priestley, 1986; Delvoye et al., 1986; Grillery et al., 1986; Guidry and Grinnell, 1985,1986 and 1987). Most of the works on collagen gel contraction treated the process as a simple step. Therefore, the results were mostly shown by the degree of contraction at a certain period of time or the qualitative comparsion of pattern of time course curve in which the lag phase was not always shown. However, the collagen gel contraction appears to result from a consequence of complicated interactions between collagen and cell and hence, as shown in Fig. I-1, I-4, and I-5, the time course of gel contraction is not expressed by a simple curve. Qualitative analysis reported previously seemed to have misled the effects of conclusion about certain factors which were clearly observed in the present study such as the effect of PDL (Fig. I-4 and Table I-3) and cytochalasin D (Fig. I-5). Degree of collagen gel contraction determined at a point of one or two days of culture would have shown that fibroblasts of high PDL contracted collagen gels much faster than cells of low PDL (compare the gel size at day one or two

in Fig. I-4). The same data might give a result that no difference in gel contraction between high PDL and low PDL of cells was observed when the contraction was compared only at the end of a 4-day period of culture.

The present study on the collagen gel contraction phenomenon has been directing toward a more quantitative analysis. The contraction curve was treated quantitatively by dividing the process into three phases which are characterized by kinetic parameters. The previous reports did not mention lag phase explicitly. However, it is clear that in the present results the lag phase in the contraction process can not be negligible. Distinct appearance of the lag phase in the present experiments may be due to the following experimental procedures or conditions; 1), As shown in Fig. I-1, decrease in cell number in collagen gel ($1x10^4$ cells/ml) which prolonged the lag phase significantly; 2), Increase in measuring points of gel diameter, particularly at the initial 48 h after starting the gel culture; 3), As described in Materials and Methods, detachment of collagen gel from plastic dish on passing 3 h after start of culture which is a requisite for maximizing reproducibility of the measurement of lag phase.

Factors examined include cell number, serum concentration, culture temperature, collagen concentration, donor age of fibroblasts, PDL of fibroblasts, and concentration of cytoskeleton-affecting drugs. The quantitatively expressed parameters can be used to test whether the three phases arbitrarily divided according to the apparent curves are correlated with one another. When a correlation exists, it may suggest that two phases are governed by a common mechanism in cell-collagen interactions. A whole set of the parameters for the second and the third phase in the present study was plotted in Fig. I-3. The rate of contraction (%/h) in the second phase vs. the reciprocal percentage of gel size on day 6 in the third phase is represented by either one of the straight lines. The close

relation under a wide range of conditions suggests that the second phase and the third phase of collagen gel contraction might be governed by a common mechanism such as a force strength exerted by the cells on collagen. In fact, Guidry and Grinnell (1986) have suggested that collagen gel reorganization by centrifugation resembled collagen gel contraction by cells, at least superficially. An external force such as centrifugal one on collagen gel can be a common determinant of parameters for contracting rate and gel size contracted when collagen gel was spun down. The force may cause collagen fibril rearrangement which results in gel contraction at a constant rate when resistant force from the gel is principally frictional. When the collagen fibrils were rearranged to a limit where the resistant force is balanced with the force exerted by cells, the size of gel would not change, which may represent the third phase.

On the other hand, the relation between the parameters for the first lag phase and those for the second phase is not so simple. With the first group of factors such as cell number, serum concentration and culture temperature, the lag time is closely correlated to the rate of contraction (Fig. I-2). However, under the conditions of the second group of factors such as different concentrations of collagen and low doses of nocodazole or colcemid (Table I-1 and I-4), the lag phase was not changed but the second phase was repressed. Increasing PDL of fibroblasts and cytochalasin D at a low dose are classified to the third group which caused a reduced lag time but no change or retardation in the second phase. Thus, under these conditions, the length of lag phase is independent of the contracting rate of the following phase. The lag phase involved a process which is independent of the later two phases. The length of lag phase is strongly dependent on collagen-cell interaction. It has been shown qualitatively that the contraction process correlates with cell spreading in collagen gel by comparing between normal and abnormal fibroblasts (Buttle and Ehrlich, 1983; Ehrlich et

al., 1983; Delvoye et al., 1986). The present results quantitatively demonstrated that the lag phase of contraction was closely correlated to the elongation process in morphological change of fibroblasts in collagen gel (Fig. I-7), suggesting that the lag phase is governed by cytokinetical activity. Thus, the effects of various factors on collagen gel contraction should be characterized by separating the process at least into two different phases; the lag phase and the later contracting phase(s).

Tomasek and Hay (1984) have reported in detail the role of cytoskeleton in the collagen-induced morphological change of corneal fibroblasts within collagen gel. Either cytochalasin D or nocodazole (more than 1 μg/ml) was inhibitory, indicating that both the microtubeles and the actin microfilaments are required for elongation process of fibroblasts. It has also been reported that the effect of cytochalasins at low doses (less than 2 μg/ml cytochalasin B or 0.25 μg/ml cytochalasin D) was not always similar to those at high doses. When the spreaded 3T3 cells on plastic were treated with cytochalasins at a low dose, the cells undergo elongation, but become arborized and rounded up at high doses of cytochalasins (Atlas and Lin, 1978). Domnina et al. (1982) have reported that low doses of cytochalasins did not inhibit the outgrowth and maintenance of lamellas at the cell periphery of fibroblasts on plastic. The previous works suggest that the effects of cytochalasins at low doses on the state of actin may be different from those at high doses. Bell et al. (1979) and Ehrlich et al. (1986) have reported that functional integrity of the cytoskeleton was essential, in that the collagen gel contraction was completely inhibited by high doses of cytochalasin B (more than 5 μg/ml) and colcemid (0.36 μg/ml). However, they did not examine the effects of these drugs at low concentrations as employed in the present study on the collagen gel contraction. The low doses (0.03 to 0.1 μg/ml) of cytochalasin D, contrary to the high doses, induced the reduction of lag time in gel contraction as well

as earlier elongation in fibroblast shape from spherical. The low dose of cytochalasin D may help cytoskeleton dynamics through its influence on actin. The initiation of actin polymerization which was observed in vitro by addition of cytochalasins at a low dose may or may not be related to this effect on cell elongation (Brenner and Korn, 1980; MacLean-Fletcher and Pollard, 1980). Low dose of nocodazole or colcemid (less than 0.03 μg/ml) did not affect the lag phase. However, the subsequent rapid contraction of collagen gel in the second phase was completely prevented by cytochalasin D, nocodazole, or colcemid at a concentration of 0.1 μg/ml. The results suggest that the initiation of collagen gel contraction as well as the elongation of cells are accelerated by acting on the state of the actin in cytoskeleton of suspended spherical cells, while the contractile force of fibroblasts requires both microtubules and actin microfilaments. The results are consistent with the close correlation between the timing of initiation of gel contraction and that of elongation of the cellular shape (Fig. I-7) and also confirm the dissociation of lag phase from contraction phase.

With increasing PDL, fibroblasts have caused the reduction in the lag time of gel contraction and the acceleration of elongation of cell shape. The following two phases of contraction were unchanged between low PDL and high PDL. The result is suspected that the state of cytoskeleton of fibroblasts was altered with increasing PDL. It has been reported that in in vitro senescent fibroblasts (high PDL) cultured on plastic, microtubules, intermediate filaments, and actin microfilaments became not only enriched but also increasingly organized (Van Gansen et al., 1979; Wang, 1984). The enriched and highly organized cytoskeletons might induce the acceleration of morphological change of fibroblasts in collagen gels from spherical to elongate. This phenomenon was clearly observed in collagen gel, while it was not discernible on plastic. The 15th PDL and 45th PDL fibroblasts attached,

flattened and spreaded rapidly at an indistinguishable speed on plastic (unpublished data).

Kinetic parameters of collagen gel contraction are characteristic for cell types. The effect of factors on gel contraction by foreskin fibroblasts were similar to those by skin fibroblasts (Fig. I-2, I-3 and I-4, and Table I-3). However, under the same culture condition, human foreskin fibroblasts from a 0-year-old male showed a retardation in lag phase and a decreased rate of contraction as compared with fibroblasts isolated from the skin of different donor ages including fetal skin fibroblasts and skin fibroblasts from 1-year-old male (Fig. I-2, I-3 and Table I-2). Mouse 3T3 established cell lines showed significantly smaller parameters in the later two phases of contraction than dermal fibroblasts (Nishiyama, et al., 1985). The kinetic parameters might be a potential index for fibroblasts from different tissues or animal species.

Table I-1. Effect of collagen concentration on collagen gel contraction

Initial concentration of collagen (mg/2ml)	Lag time (h)	Rate of contraction (%/h)	Percentage contracted on day 6 (%)
0.6	30	1.9	79 ± 1
1.0	30	1.6	71 ± 1
2.0	30	0.66	52 ± 2
3.0	34	0.41	43 ± 1

HF-0 (15th PDL) populated collagen gels were made with 2.0 x 10^4 cells, different concentrations of type I collagen and 10% FBS per 2 ml in 35 mm Petri dishes. Each parameter was calculated from the time course curves of gel contraction by the measurement of 4 gels.

Table I-2. Effect of donor ages of human skin fibroblasts on collagen gel contraction

Fibroblasts Abbreviation	PDL	Lag time (h)	Rate of contraction (%/h)	Percentage contracted on day 6 (%)
HEF-4	11	13	3.3	80 ± 1
HEF-5	12	13	3.0	78 ± 1
HF-1	9	15	3.0	77 ± 1
HF-30	9	15	3.5	77 ± 1
HF-41	8	15	3.5	76 ± 0
HF-60	8	15	3.8	76 ± 1
HF-69	8	15	3.8	75 ± 1
HF-71	8	15	3.5	78 ± 0

Each fibroblast populated collagen gel was made with 2.0 x 10^4 cells, 1.0 mg of type I collagen and 10% FBS per 2 ml in 35 mm Petri dishes. Each parameter was calculated from the time course curves of gel contraction by the measurement of 3 or 4 gels.

Table I-3. Effect of PDL of HF-30 on collagen gel contraction

PDL	Lag time (h)	Rate of contraction (%/h)	Percentage contracted on day 6 (%)
5	14	3.8	73 ± 2
10	14	3.8	74 ± 1
18	7	3.8	73 ± 1
28	5	4.1	72 ± 0

HF-30 populated collagen gel was made with 2.0×10^4 cells, 1.0 mg of type I collagen and 10% FBS per 2 ml in 35 mm Petri dishes. Each parameter was calculated from the time course curves of gel contraction by the measurement of 4 gels.

Table I-4. Effect of nocodazole or colcemid on collagen gel contraction

Concentration (μg/ml)	Lag time (h)	Rate of contraction (%/h)	Percentage contracted on day 6 (%)
Nocodazole			
0	32	1.6	73 ± 1
0.001	32	1.6	73 ± 1
0.01	32	1.3	66 ± 1
0.03	32	0.42	20 ± 1
0.1	--	0	0
Colcemid			
0.001	32	1.6	72 ± 2
0.01	32	0.94	56 ± 3
0.03	32	0.28	11 ± 2
0.1	--	0	0

HF-0 (15th PDL) populated collagen gels were 2.0×10^4 cells, 1.0 mg of type I collagen and 10% FBS per 2 ml in Petri dishes. Drugs were present in culture medium during the experiment. Media were renewed every 2 days. Each parameter was calculated from the time course curves of gel contraction by the measurement of 4 gels.

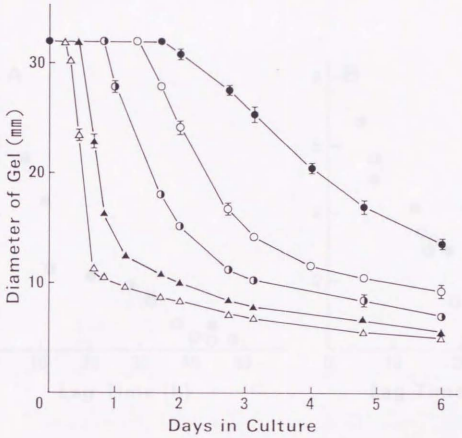

Fig. I-1. Time course curves of collagen gel contraction with different number of human foreskin fibroblasts.
HF-0 (15th PDL) populated collagen gels were made with different number of cells, 1 mg of type I collagen and 10%FBS per 2 ml in 35 mm Petri dishes. The inner diameter of the dish was 32 mm. Initial number of cells was 1.0×10^4 (●), 2.0×10^4 (○), 4.0×10^4 (◑), 10×10^4 (▲) and 20×10^4 (△) cells/2 ml. Media were renewed every 2 days. Each point represents the mean of quadruplicate determinations ± SD.

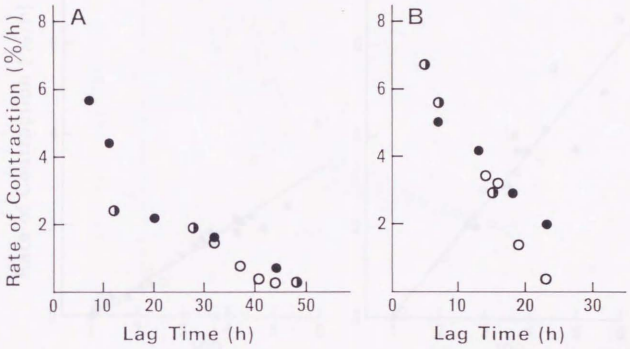

Fig. I-2. Relationship between lag time and rate of contraction under the different conditions of cell number, FBS concentration or culture temperature.
Lag time or rate of contraction was calculated from the time course curves of gel contraction by HF-0 (A) or HF-30 (B). Culture conditions : ● cell number (1.0×10^4 to 20×10^4 cells) ; ○ FBS concentration (0.5 to 20%) ; ◑ culture temperature (32.5 to 39.5 °C).

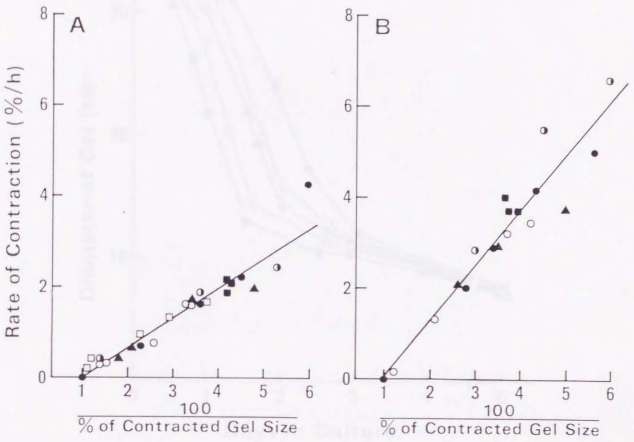

Fig. I-3. Relationship between rate of contraction and contracted gel size on day 6.

Rate of contraction and percentage of contracted gel size on day 6 were calculated from the time course curves of gel contraction by HF-0 (A) or HF-30 (B). Factors affecting the gel contraction: ● cell number; ○ FBS concentration; ▲ type I collagen concentration; ◑ culture temperature; ■ different PDL of cells; □ addition of nocodazole or colcemid.

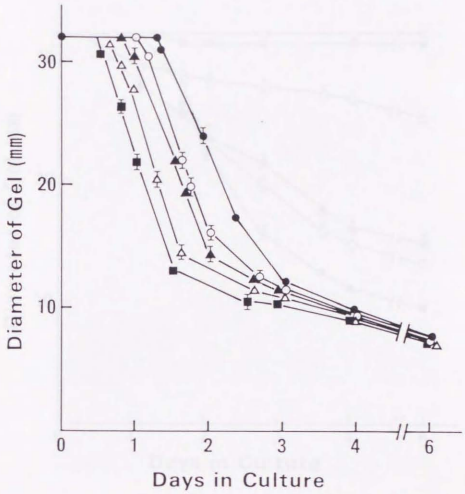

Fig. I-4. Contraction of collagen gels by human foreskin fibroblasts with different PDLs.
HF-0 populated collagen gels were made with 2.0 x 10^4 cells, 1.0 mg of type I collagen and 10% FBS per 2 ml in 35 mm Petri dishes. PDL of HF-0: ● 15th; ○ 20th; ▲ 30th; △ 40th; ■ 46th. Media were renewed every 2 days. Each point represents the mean of triplicate determinations ± SD.

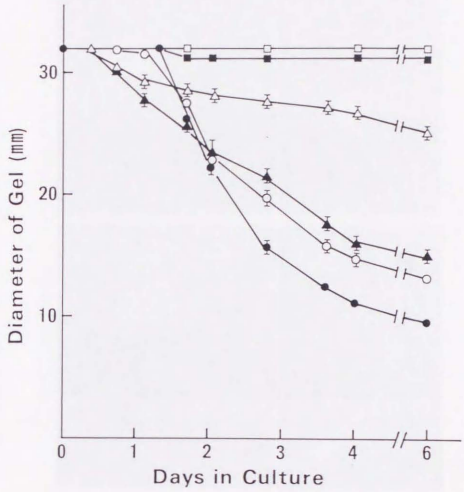

Fig. I-5. Effect of cytochalasin D on collagen gel contraction.

HF-0 (15th PDL) populated collagen gels were made with 2.0 x 10^4 cells, 1.0 mg of type I collagen and 10% FBS per 2 ml in 35 mm Petri dishes. Media containing cytochalasin D at a concentration of 0 (●), 0.01 (○), 0.03 (▲), 0.1 (△), 0.3 (■) or 1.0 μg/ml (□) were renewed every 2 days. Each point represents the mean of quadruplicate determinations ± SD.

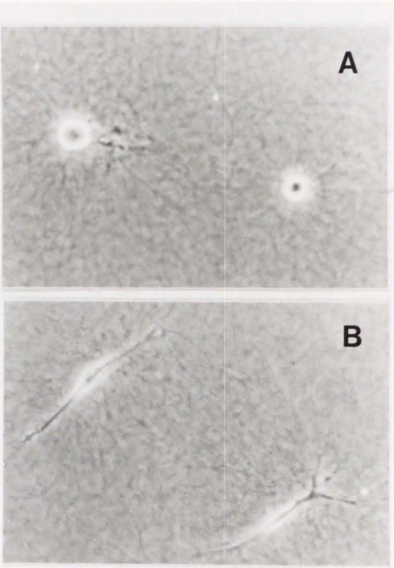

Fig. I-6. Human foreskin fibroblasts in collagen gel after treatment with cytochalasin D at a concentration of 0.1 µg/ml. A: HF-0 (15th PDL) at 10 h after the start of collagen gel culture shows spherical shape. B: HF-0 (15th PDL) at 10 h after the start of collagen gel culture with cytochalasin D shows elongated appearance. x242

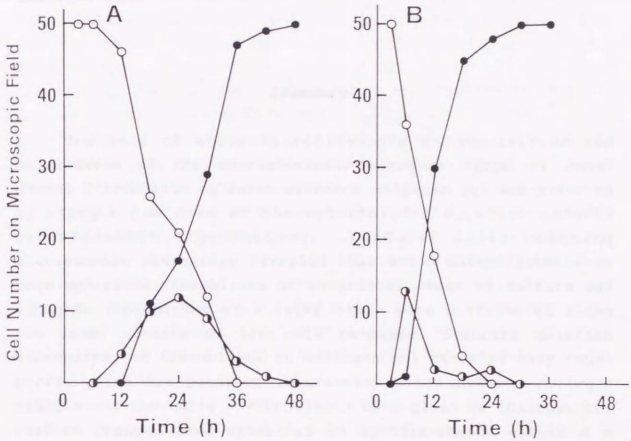

Fig. I-7. Morphological change of human foreskin fibroblasts in collagen gel observed with video-microscope.

HF-0 (15th PDL) (A) or HF-0 (45th PDL) (B) populated collagen gels were made with 2.0 x 10^4 cells, 1.0 mg of type I collagen and 10% FBS per 2 ml in 35 mm culture dishes. Magnified views of cells in collagen gels were recorded on video tape for 3 days. Three to 5 cells in the field were observed in an experiment. The cells initially remained spherical (○), took an intermediate shape (◑) and then became elongate in shape (●). Cell numbers of each type of cell shape were counted on video tape at the indicated periods of time.

Chapter II.

Morphology of Human Dermal Fibroblasts in Three-Dimensional Collagen Gel.

[Summary]

The role of actin microfilaments in acquisition and maintenance of the characteristic elongate shape of human dermal fibroblasts cultured within a collagen gel was examined by using a low dose of the cytochalasin D, which affects microfilament organization. Confocal laser scanning fluorescence microscopy revealed that actin microfilaments in both spherical fibroblasts at an initial stage of culture and elongate fibroblasts at a later stage were distributed along the inner surface of the cell membrane. Scanning electron micrographs of fibroblasts in collagen gel revealed many rough picks, which are putative interaction sites between collagen fibrils and the cells. Fibroblasts on a glass or collagen gel surface changed from spherical to spindle-shaped within 6 h after the start of culture, while within collagen gel, the morphological change was markedly retarded, and occurred at 30 h or later. This retardation was antagonized by a low dose of cytochalasin D (0.2 μM). The treatment induces disorganization of actin microfilaments on the one hand, but organization of microtubules or intermediate filaments to scaffold the elongate shape on the other hand. The extremely elongate cell shape once formed in collagen gel was not affected by cytochalasin D. We hypothesize that the actin microfilaments form the spherical scaffold or prevent acquisition of elongate shape of fibroblasts at an initial stage of culture in collagen gel, but do not participate in maintenance of the elongate shape of the cell at a later stage.

1. Introduction

The extracellular matrix plays an important role in regulating the cytoskeletal organization, morphology and other functions of fibroblasts (Chapter I; Kleinman et al., 1981; Tomasek et al., 1982; Tomasek and Hay, 1984; Nusgens et al., 1984; Colige et al., 1988). The study in this chapter was focused on the influence of the three-dimensional collagen matrix on cell morphology and cytoskeletal organization. Fibroblasts in collagen gel rapidly become attached to collagen fibrils by interaction of the cell surface receptors directly with collagen or indirectly with collagen via fibronectin (Wayner and Carter, 1987; Carter and Wayner, 1988; Akiyama et al., 1990; Gullberg et al., 1990). Eventually the cells become extremely elongate in shape (Tomasek and Hay, 1984; Nishiyama et al., 1988) and contract the collagen gel (Bell et al., 1979; Guidry and Grinnell, 1985; Nishiyama et al., 1988; Gullberg et al., 1990). The elongate shape of fibroblasts is one of the specific characteristics of the cells cultured in collagen gel as distinct from the cells cultured on the gel. This shape change is considered to be closely correlated to the cytoskeletal organization as described in Chapter I.

Tomasek and Hay (1984) have reported in detail the role of cytoskeleton in the collagen-induced morphological change of embryonic chick corneal fibroblasts within collagen gel. They concluded that both intact actin microfilaments and microtubules are required for extensive elongation of corneal fibroblasts within collagen gel, since either cytochalasin D, which is known to act primarily on the state of actin, or nocodazole, which is known to disrupt microtubules, was inhibitory. The results were obtained by using a high dose of cytochalasin D (more than 2 μM). However, it has been reported that the effect of cytochalasins at low doses (less than 0.5 μM cytochalasin D) is not always similar to that at high doses (Atlas and Lin, 1978; Domnina et al., 1982). For example, low

doses of cytochalasins did not affect the gross spread of fibroblasts on plastic and did not inhibit the outgrowth and maintenance of lamellas at the cell periphery, while fibroblasts became arborized and rounded in shape at high doses of cytochalasins. We have also reported that low doses (0.06 to 0.2 μM) of cytochalasin D, unlike high doses, induced the elongation of fibroblasts within collagen gel at an earlier timing (Chapter I).

In the present study, I have examined the cell morphology and cytoskeletal organization, particularly actin microfilaments, of human dermal fibroblasts induced by culture within a three-dimensional collagen gel as compared to those of a culture on a two-dimensional surface of collagen gel or on glass. By using a low dose of cytochalasin D, I obtained a different result on the role of actin microfilaments from that described by Tomasek and Hay (1984). That is, the time required for the morphological change of fibroblasts within collagen gel from spherical to elongate was shortened by treatment with a low dose of cytochalasin D. The treatment caused disorganization of actin microfilaments and organization of a cytoskeleton component other than actin, resulting in accelerated elongation of fibroblasts. In addition, the low dose of cytochalasin D did not change the elongated shape of fibroblasts which had already been formed, even though it disrupted actin microfilaments.

2. Materials and Methods

Cell culture

Fibroblasts were isolated from human foreskin of a 0-year-old male (HF-0) and from human skin of a 30-year-old female (HF-30) and a 41-year-old male (HF-41) obtained during plastic surgery, as described in Chapter I. Cultures at the 4th population doubling level (PDL) were frozen and kept in

liquid nitrogen. HF-0 from the 15th PDL to 18th PDL and HF-30 and HF-41 from the 8th PDL to 10th PDL were used throughout the experiments.

Preparation of collagen substrata and cell cultures

A solution of pepsin-treated type I collagen (porcine tendon) in dilute HCl (pH 3.0) at a concentration of 3.0 mg/ml was obtained from Nitta Gelatin Co. (Osaka).

For culture with Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical Co., Tokyo) containing 10% fetal bovine serum (FBS, GIBCO Labs., New York) on three-dimensional gels of collagen fibrils, 0.2 ml aliquots of a solution (collagen-medium solution) obtained by rapidly mixing 3 ml of collagen solution (3 mg/ml), 1.5 ml of 3x concentrated DMEM with sodium bicarbonate, penicillin and streptomycin, 0.5 ml of FBS, and 4 ml of 10% FBS-DMEM were pipetted onto 15-mm coverslips in 35-mm petri dishes and incubated for 1 h at 37 °C to form a gel. Two ml of the cell suspension in 10% FBS-DMEM (1×10^4 cells/ml) was placed on a plastic dish including a coverslip and incubated at 37 °C for the indicated time.

Incorporation of cells within the three-dimensional collagen gel was performed by rapidly mixing a cell suspension with the collagen-medium solution (10% FBS-DMEM), then plating the mixture (0.2 ml) to give a final cell density of 1×10^4 cells/ml and a collagen concentration of 1 mg/ml onto 15-mm coverslips in 35-mm petri dishes. Each dish was incubated for 1 h at 37 °C to form the gel before the addition of 2 ml of culture medium.

Microscopy of living fibroblasts

Fibroblasts cultured on collagen gels or within collagen gels were examined with a phase-contrast microscope or a differential interference contrast microscope (Nikon, Tokyo). The stage was surrounded by a plastic chamber and kept under an atmosphere of 95% air and 5% CO_2 and at 37 °C by applying heated air intermittently. Magnified views of fibroblasts

within collagen gels were recorded on video tape for 0.1 second at intervals of 30 seconds for 3 days by using a video-recording system (Sankei Co., Tokyo).

Rhodamine-labeled phalloidin staining and indirect immuno-staining using FITC-labeled antibodies

At various times after the start of culture, the collagen gels were fixed with 4 % paraformaldehyde in PBS for 20 min, washed 3 times for 10 min with PBS, and then permeabilized in 0.5 % Triton X-100 in PBS for 10 min prior to staining. Some of the gels were incubated with rhodamine-labeled phalloidin (Molecular Probes Inc., Eugene, OR; diluted 1:10) for 2 h at room temperature in the dark, washed three times for 10 min with PBS and mounted on glass slides in 90 % glycerol in PBS. Other gels were incubated for 30 min with 1% bovine serum albumin in PBS and then incubated with anti-α tubulin monoclonal antibody (Amersham, UK) or anti-vimentin monoclonal antibody (Amersham, UK) for 1 h at room temperature, washed three times for 30 min with PBS, incubated with goat anti-mouse IgG-FITC (Kirkegaard & Perry Lab. Inc.) for 1 h at room temperature, washed three times for 30 min with PBS, and mounted on glass slides in 90 % glycerol in PBS. Fibroblasts in the gels were viewed with an Olympus AH-2 microscope or with a Bio Rad MRC-500 laser scanning confocal microscope in the fluorescence mode.

Scanning electron microscopy

Collagen gels containing fibroblasts were prepared on glass coverslips and incubated for 7 days. They were processed for a scanning electron microscopy according to the ODO (osmium tetroxide-dimethyl surfoxide-osmium tetroxide) method originally introduced by Tanaka et al. (1981 and 1984). The gels were immersed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 4 h at 4 C. They were postfixed with 0.1% osmium tetroxide (OsO_4) in the same buffer for 2 h at 4 C and reinsed twice with 0.1 M phosphate buffer. They were then

immersed in 25% and 50% dimethyl sulfoxide (DMSO) for 1 h each at room temperature. Some of them were frozen and shaved on surface with precooled razor blade in liquid nitrogen to reveal inner structures of the gels. The shaved gels were thrawed in 50% DMSO at room temperature and rinsed in 0.1% OsO_4 solution for 3 days at room temperature. The gels with or without shaving were dehydrated through a graded series of ethanol and immersed in iso-amyl acetate. They were dried in a critical point dryer (Hitachi HCP-2, Hitachi Co. Ltd., Japan) with dry ice. Dried specimens were mounted on aluminium stubs and sputter coated with platinum, approximately 10 nm thick, in an ion-coater with rotating stage (Eiko VX-10R, Eiko engineering Co. Ltd., Japan). The metal-coated specimens were observed with a field emission scanning electron microscope (Hitachi S-800, Hitachi Co. Ltd., Japan).

Other Materials

Cytochalasin D, nocodazole and colcemid were purchased from Aldrich Chemical Co. (Milwaukee, WI), Janssen (Belgium) and Wako Pure Chemical Industries (Osaka), respectively.

3. Results

3.1 Surface structure of fibroblasts cultured in a three-dimensional matrix of collagen gel

The interphase structure between collagen fibrils and human foreskin fibroblasts (HF-0) cultured on or within collagen gel was studied by scanning electron microscopy. Fibroblasts on collagen gel were flat in shape with sheet-like filopodia and a mound containing a nucleus (Fig. II-1-1a). These characteristics were essentially comparable to those of fibroblasts cultured on plastic or glass surfaces. Free

surfaces of fibroblasts were rather smooth. Occasionally, collagen fibrils were observed on the free surfaces (Fig. II-1-1a). On the other hand, fibroblasts cultured within collagen gel extended long cell processes and surrounded entirely by collagen fibrils, approximately 80 nm in diameter. Collagen fibrils were densely packed in the vicinity of fibroblasts (Fig. II-1-1b). Many rough picks (about 50-100 nm in diameter), which were attached on the cell surface of fibroblasts cultured within collagen gel, were observed in Fig. II-1-2. These rough picks gradually merged to the contour of fibroblasts. Close inspections revealed that collagen fibrils anchored to the surfaces of fibroblasts (Fig. II-1-1b and II-1-2).

3.2 Organization of actin microfilaments in fibroblasts cultured in a three-dimensional matrix of collagen gel

Fibroblasts cultured on glass, on collagen gel or within collagen gel were examined by staining with rhodamine-labelled phalloidin, which stains actin in the form of filamentous aggregates (Fig. II-2). A spindle-like flat shape of fibroblasts on glass or on collagen gel showed actin microfilament organization or stress fibers (Fig. II-2; A and B) spanning between focal regions, while the cells in collagen gel showed straight filaments running in parallel along the elongate shape (Fig. II-2C). The three-dimensional organization of actin microfilaments of the cells in collagen gel was examined by laser-scanning confocal microscopy (Fig. II-3). Actin microfilaments of the fibroblasts cultured on glass were organized mainly along the inner surface of the cell membrane in contact with the glass. In the elongated cells in collagen gel at 40 h after the start of culture, a cross-section of the bundles of actin filaments aligned in the direction of elongation showed a distribution along the inner surface of the cell membrane (Fig. II-3B).

3.3 Morphological change of fibroblasts in a three-dimensional matrix of collagen gel

In the culture of fibroblasts on glass, on collagen gel and within collagen gel, trypsinized spherical fibroblasts initially attach to the substrata and then spread. The timing of fibroblast spreading within collagen gel was examined as compared to that on glass and on collagen gel. Fibroblasts on glass and on collagen gel became spindle-like in shape within 6 h after start of culture (Fig. II-4; A and B). On the other hand, within collagen gel the morphological change of fibroblasts from spherical to elongate was markedly retarded and became apparent at 30 h or more after the start of gel culture (Fig. II-4; C and D, and Fig. II-5).

Cytochalasin D (CD), which is known to inhibit actin polymerization, induced arborization (Fig. II-6A) or rounding of the cell shape at a concentration of 2 μM when the cells were cultured on glass, on collagen gel and within collagen gel. However, a lower dose of CD (0.2 μM), which did not affect the spindle-like morphology of cells cultured on glass or on collagen gel (data not shown), accelerated the morphological change from spherical shape to elongated form within collagen gel to earlier than 15 h after the start of gel culture (Fig. II-5 and Fig. II-6B). Elongated fibroblasts induced by the low dose of CD reverted to spherical form upon either removal of CD from the medium or addition of nocodazole at a concentration of 0.1 μM, which is sufficient to disrupt microtubules (data not shown).

A low dose of cytochalasin A (0.2 μM), B (2 μM), C (0.2 μM) or E (0.02 μM) was also effective in antagonizing the retardation of morphological change of fibroblasts within collagen gel (data not shown), resulting in formation of the elongate cell shape at an earlier timing than without the drug.

3.4 Organization of fibroblast cytoskeletons in a three-dimensional matrix of collagen gel after treatment with the low dose of cytochalasin D

Cytoskeleton organizations in elongated fibroblasts within collagen gel induced by treatment with the low dose of CD were examined. In the spherical cells in collagen gel before elongation, actin filaments were distributed along the inner surface of the cell membrane, as shown in Fig. II-7; A and B, as if actin filaments form the spherical scaffold of fibroblasts at the initial stage of culture in collagen gel. In the elongated cell shape induced by CD treatment at 10 h, a time when elongation did not take place in the absence of CD treatment, actin filaments were disrupted from bundles to fragments (Fig. II-8A). The organization of microtubules (Fig. II-8C) or intermediate filaments (Fig. II-8E) was regular as observed by indirect immunostaining with anti-tubulin Ig G or anti-vimentin Ig G. The organizations of microtubules and vimentin intermediate filaments induced by CD treatment were indistinguishable from those observed in elongate cells in the control culture at 40 h without CD (Fig. II-8; D and F). The addition of CD did not change the extremely elongated cell shape observed in the collagen gel at 40 h after the start of culture, though it disrupted actin microfilaments from bundles to fragments (Fig. II-8B) as compared to the straight filaments in elongate cells in control culture (Fig. II-2C and Fig. II-3).

4. Discussion

It is reported here that morphological change of human fibroblasts cultured within collagen gel was markedly retarded as compared with that on glass or on collagen gel. The retardation was antagonized by a low dose of cytochalasins,

which disrupted bundles of actin filaments to fragments. The present observations suggest that acquisition of elongate shape of human fibroblasts cultured within collagen gel may occur through the following sequential steps: 1) actin microfilaments maintain the spherical scaffold of trypsinized spherical fibroblasts at the initial stage of culture within collagen gel through interactions with surrounding collagen fibrils; 2) fragmentation of actin microfilaments occurs, disrupting the spherical scaffolding; 3) organization of a cytoskeleton other than the actin microfilaments induces and maintains an elongated cell shape through cell attachment to collagen fibrils on all sides of the cell membrane; 4) maximization of the cell-collagen attachments results in the extremely elongate shape. Direct involvement of actin microfilaments in maintenance of the characteristic elongate shape of human fibroblasts within collagen gel is most unlikely, since the addition of a low dose of cytochalasin D did not affect the elongate shape even though it appreciably disrupted actin microfilaments.

Fibroblasts showed a characteristic morphology and actin microfilament organization within collagen gel, distinct from those on collagen gel or on glass, as shown in Figs. II-2, II-3 and II-4. It should be noted that the cells within collagen gel have the entire surface surrounded by collagen fibrils (Fig. II-1; 1b and 2), while the cells on collagen gel have only the lower surface of the cells in contact with collagen fibrils, and the upper side is free from substrata (Fig. II-1-1a). Therefore, the difference in cellular shape and actin microfilament organization could have resulted from the distribution of cell-collagen interaction sites on the cell. Fibroblasts interacting with the three-dimensional network of collagen fibrils showed repression in growth rate, in mitogenic response to growth factors and in collagen synthesis as compared to cells cultured on the surface of collagen gel or on glass (Chapter III and IV; Nusgens et al., 1984; Mauch et al., 1988; Nakagawa et al., 1989; Nishiyama et

al., 1989 and 1990). The repression of cellular activities caused by the three-dimensional collagen gel might be correlated to the cellular morphology and cytoskeletal organization.

We next examined the effect of a low dose of cytochalasin D on the induction of morphological change from spherical to elongate shape of fibroblasts cultured in collagen gel. Actin microfilaments of fibroblasts spherical in shape at the initial stage of collagen gel culture were distributed along the inner surface of the cell membrane (Fig. II-7). At this stage, intermittent projections of filopodia from the surface of trypsinized spherical fibroblasts were observed by using the video-recording system (unpublished data), as if the fibroblasts were attempting to bind to collagen fibrils. It has been reported that the extracellular matrix receptors in the cell membrane, integrins, are remarkably resistant to digestion with protease (Carter and Wayner, 1988). In collagen gel, the initially spherical cells are able to interact with collagen fibrils on all sides of the cell membrane. This interaction might stabilize the actin microfilaments organized along the inner surface of the spherical cell, which might inhibit the elongation process of fibroblasts in collagen gel. In the control culture, fibroblasts became elongate in shape only at 30 h or later (Fig. II-5), while treatment with cytochalasin D at a low dose accelerated the elongation process, involving the organization of a cytoskeleton component other than actin. In contrast to the low dose of cytochalasins, high dose of cytochalasins completely inhibited the elongation of fibroblasts and induced arborization or rounding up of cell shape. Therefore, the high dose of cytochalasins may prevent not only the organization of actin filaments but also that of other cytoskeleton. In fact, spherical fibroblasts within collagen gel upon treatment with nocodazole, which is known to disrupt microtubules, can not become elongate in shape and still remain spherical form. The low dose of cytochalasins might regulate cytoskeleton dynamics

through its specific action on actin microfilaments. The intriguing possibility arises that for elongation in the control culture without cytochalasins, fibroblasts themselves may produce a factor with cytochalasin-like activity to initiate the elongation process. If so, the prolonged lag time before morphological change of fibroblasts in the control culture might reflect the period required for the production of the factor.

Based on the present results, we propose that: 1) actin microfilaments interfere with the acquisition of elongate shape of fibroblasts cultured within collagen gel by maintaining the spherical scaffold structure at the initial stage of culture, and 2) actin microfilaments do not participate in maintenance of the extremely elongated shape which fibroblasts acquire in collagen gel after a sufficient period of time. In addition, we have also observed that human fibrosarcoma HT1080 cells became elongate in shape within collagen gel in spite of the disruption of actin microfilament organization as observed in fibroblasts treated with a low dose of CD. We conclude, therefore, that the elongate shape of fibroblasts, which is one of the specific characteristics of the cells, is maintained by the interactions in a linkage of extracellular matrix (ECM)--ECM receptors--cytoskeleton other than the well-known linkage of fibronectin--fibronectin receptor (one of the integrin family)--(talin, vinculin, α-actinin)--actin filaments (Horwitz et al., 1986; Burridge, 1988; Alberts et al., eds., 1989-a).

The effects of collagen on cells are thought to be mediated through specific cell surface recepters. In contrast to the other receptors, many different putative collagen receptor molecules have been reported, but none have yet been characterized in detail. Recently, a collagen-binding protein termed "CRIII") has been identified by Carter and Wayner (1988). The CRIII appears to be a transmembrane protein often co-localizing with vimentin. Therefore, this collagen receptor is a leading candidate for a possible transmembrane

link between collagen and a specific cytoskeletal protein, vimentin. If so, one possibility is that the elongate shape of fibroblasts within collagen gel is mainly maintained in a specific linkage of collagen fibrils--collagen receptor --vimentin (intermediate filaments). Further analyses will be necessary to identified conclusively this interaction between extracellular and intracellular organizations.

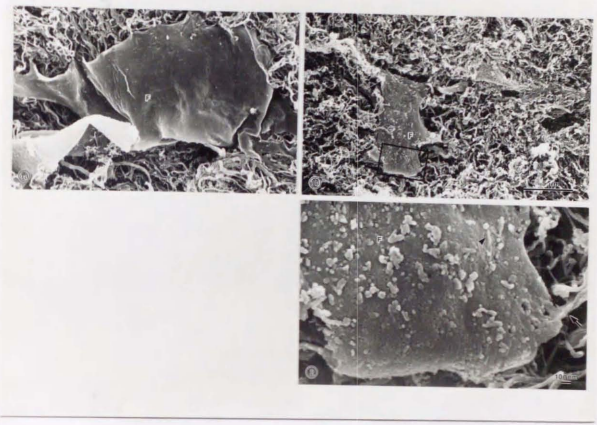

Fig. II-1. Scanning electron micrographs of human fibroblasts. HF-0 was cultured on collagen gel or within colagen gel for 7 days. **1a:** cell surface of HF-0 (F) cultured on collagen gel. **1b:** fracture plane of collagen gel containing HF-0 (F). **2:** higher magnification of inscribed area in **1b.** Rough picks are attached on the cell surface in collagen gel and merged to the contour of cell (arrowhead). Collagen fibrils (arrow) anchor the cell surface.
(Electron microscopy was examined by Dr. E. Adachi in Osaka University Medical School.)

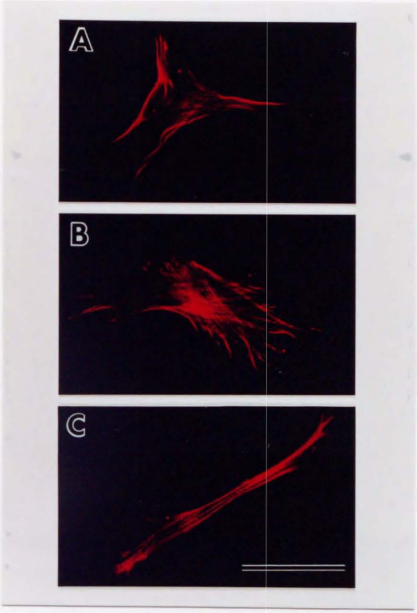

Fig. II-2. Actin microfilament organization in human fibroblasts under various conditions. HF-0 was cultured for 40 h on glass, on collagen gel or within collagen gel and the distribution of actin microfilaments was examined by staining with rhodamine-labeled phalloidin as described in Materials and Methods. Bar, 100 μm. **A:** HF-0 on glass. **B:** HF-0 on collagen gel. **C:** HF-0 within collagen gel.

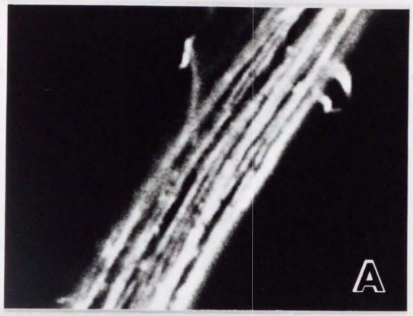

Fig. II-3. Actin microfilament organization of human elongated fibroblasts in collagen gel examined by confocal microscopy. HF-0 was cultured for 40 h in collagen gel and the distribution of actin microfilaments was examined by staining with rhodamine-labeled phalloidin. Bar, 10 μm. **A:** microscopic tomography of actin microfilaments. **B:** vertical tomography of actin microfilaments.

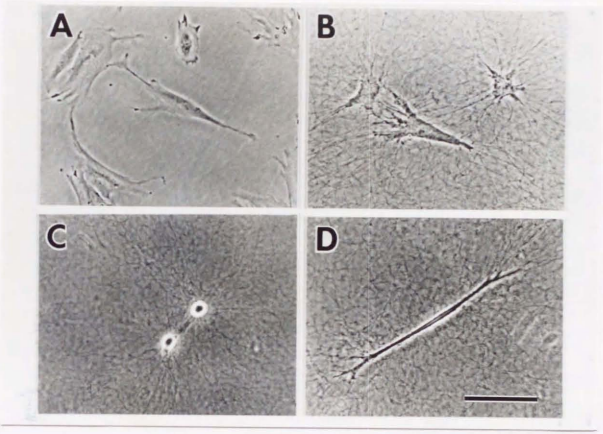

Fig. II-4. Morphology of human fibroblasts cultured on glass, on collagen gel or within collagen gel. HF-0 was cultured on glass, on collagen gel or within collagen gel and the morphology of HF-0 was examined with a phase-contrast microscope. Bar, 100 μm. **A:** on glass for 6 h. **B:** on collagen gel for 6 h. **C:** in collagen gel for 15 h. **D:** in collagen gel for 36 h.

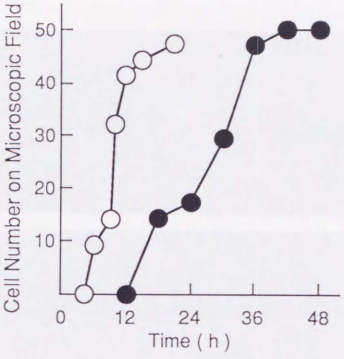

Fig. II-5. Elongation of human fibroblasts in collagen gel upon treatment with cytochalasin D at a concentration of 0.2 μM. HF-0 populated collagen gel was made with 2.0 x 10^4 cells, 1.0 mg of type I collagen and 10 % FBS per 2 ml in 35 mm culture dish in the absence of cytochalasin D (●) or in the presence of cytochalasin D (○). Magnified views of cells in collagen gel were recorded on video tape for 48 h. Three to five cells in the field were observed in an experiment. The cells initially remained spherical, took an intermediate shape and then became elongate in shape. Cell numbers of elongated shape were counted on video tape at indicated periods of time.

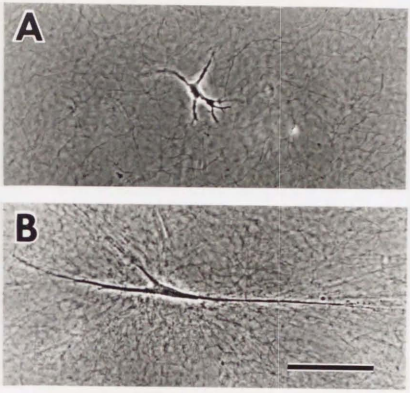

Fig. II-6. **Effect of cytochalasin D on morphological change of human fibroblasts within collagen gel.** **A:** HF-0 at 15 h after the start of collagen gel culture with a high dose of cytochalasin D (2 μM) shows an arborized shape. **B:** HF-0 at 15 h after the start of collagen gel culture with a low dose of cytochalasin D (0.2 μM) shows an elongated shape. Bar, 100 μm.

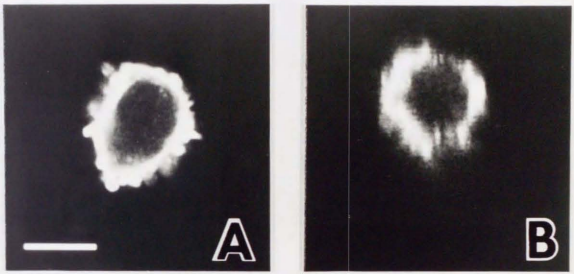

Fig. II-7. **Actin microfilament organization of spherical fibroblasts within collagen gel observed by confocal microscopy.** HF-0 was cultured for 3 h in collagen gel and the distribution of actin microfilaments was examined by staining with rhodamine-labeled phalloidin. Bar, 10 μm. **A:** microscopic tomography of actin microfilaments. **B:** vertical tomography of actin microfilaments.

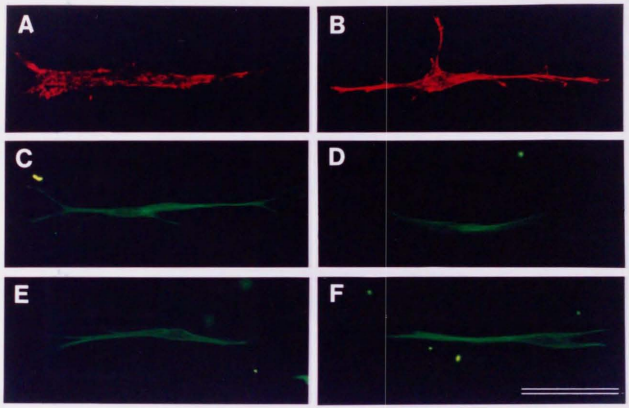

Fig. II-8. Cytoskeleton organizations of human elongated fibroblasts within collagen gel upon treatment with a low dose of cytochalasin D. HF-0 was cultured in collagen gel for 10 h with cytochalasin D (0.2 μM) (**A, C** and **E**) or for 40 h without cytochalasin D (**B, D** and **F**), followed by further incubation for 3 h with cytochalasin D (0.2 μM) (**B**). The distributions of cytoskeletons were examined by staining with rhodamine-labeled phalloidin (**A** and **B**) or by immunostaining with anti-tubulin IgG (**C** and **D**) or anti-vimentin IgG (**E** and **F**). Bar, 100 μm.

Chapter III

Growth Rate of Human Dermal Fibroblasts Cultured within Three-Dimensional Collagen Gel

[Summary]

Effect of reconstituted type I collagen gel on growth behavior of human dermal fibroblasts was examined. A prolonged lag period was observed by culture either on the surface of three-dimensional collagen gel ("on-gel") or within the gels ("in-gel") as compared with the culture on plastic dishes. The rate of cell proliferation in logarithmic phase growth was repressed by the culture "in-gel" but not by the culture "on-gel". The differential growth rates between "in-gel" culture and "on-gel" culture should be ascribed to difference in distributions of interacting sites between cell and collagen fibrils. The repression of cell growth was more marked in a contracting collagen gel which contains higher density of collagen fibrils. The cell density in contracted gel per unit cross-section area was found to be much lower than that of confluent monolayer culture. These results suggest that the repression of cell growth by collagen fibrils in the three-dimensional gels is not due to direct cell-cell contact, but due to the distribution and number of contact sites between a cell and collagen fibrils.

1. Introduction

From the results in Chapter II, we presume that the distinctive effects of "in-gel culture", where the cells have the entire surface surrounded by the collagen fibrils, from "on-gel" culture, where the cells have only the lower surface of the cells in contact with collagen fibrils and the upper side is free from substrata on the cell morphology and cytoskeleton organization will be also observed on other cellular activities. The examination in this chapter is focused on the effect of reconstituted collagen fibrils on fibroblast growth.

In regard to fibroblast growth, Hata and Seno (1989) have reported that L-ascorbic acid 2-phosphate promotes reorganization of a three-dimensional tissue-like substance from skin fibroblasts in culture by stimulating collagen accumulation in the fibroblast layer, resulting in the stimulation of fibroblast proliferation. From the results reported by Nakagawa et al. (1989), fibroblasts cultured in collagen gel showed increase in ^{3}H-thymidine incorporation into DNA and on the contrary the cells cultured in contracting collagen gel showed reduction in ^{3}H-thymidine incorporation as compared with the cells in monolayer culture on plastic dish. It has been reported that normal fibroblasts proliferated more rapidly on plastic dishes than on the surface or within the collagen gel matrix (Schor, 1980; Schor et al., 1982; Buttle and Ehrlich, 1983). Elsdale and Bard (1972) reported that cells cultured on collagen substrata showed reduction in ^{3}H-thymidine incorporation. Yoshizato et al. (1984 and 1985) have shown that ^{3}H-thymidine incorporation was suppressed in the cells which were cultured on native collagen-coated dishes, but not in the cells cultured on denatured collagen-coated dishes. Sarber et al. (1981) showed that human fibroblasts in contracting collagen lattice ceased to incorporate ^{3}H-thymidine into DNA after four days of culture. All the above mentioned previous reports can be summarized essentially as

one conclusion that native collagen influences fibroblast growth, though the contradictory effects of collagen on cell growth were observed. Thus, the effect of collagen gel matrix on cell proliferation in vitro still appears to be ambiguous, whether it is essentially repressive or not, and to be uncertain in some detailed points.

In this chapter, we have examined the effect of collagen on fibroblast proliferation with giving attention to following points. First, cell growth curves in culture are not so simple. These are generally expressed by two empirical parameters; a time length of the period prior to logarithmic growth (generally less than 24 h after the start of culture) and a growth rate during logarithmic phase. In most previous reports, the effect of collagen was examined by ^{3}H-thymidine incorporation at an early stage by 48 h after the start of culture. Examination of growth curves was reported in few reports. We found, however, the retardation of morphological change of fibroblasts within collagen gel at an early stage which is induced by the interaction between a cell and collagen in Chapter II. It is also found that the lag phase of collagen gel contraction should be separated from the later contracting phase, since each phase is governed by two different cytokinetical activities. Thus, the effects of collagen matrix on cell growth should be examined by separating the growth curves into the lag phase at an initial stage and the logarithmic growth phase.

Second, in some previous reports, cellular behavior of "in-gel" culture has not been clearly or quantitatively distinguished from that of "on-gel" culture. In most cases, the presence of collagen in native forms has been compared with the absence or the presence of denatured forms of collagen. The distinctive treatment of "in-gel" culture from "on-gel" culture was crucial in the effects on cellular shape and its change, particularly by cytochalasin D, of fibroblasts (Chapter I and II). Third, it was found that cell proliferation was further more strongly repressed in

contracted collagen gel than "in-gel" culture without contraction. An interpretation for the important finding is not proposed until now.

For the first and second points, the two parameters of cell growth were found to distinguish growth curves of "in-gel" culture growth from those of "on-gel" culture. The lag phase of growth curve was prolonged by the contact with collgen, while the growth rate was repressed in fibroblasts cultured within collagen gel. For the third point, we have examined three possible causes of growth inhibition by "in-gel" culture; decreased diffusion of nutrients to the cells, increased cell-to-cell contacts, particularly in the case of growth inhibition of cells in contracted gel, and high density of collagen fibrils comparable to in vivo situation, resulting in increased cell-fibril interactions. We found the last cause would be most influential.

2. Materials and Methods

Cell culture

Fibroblasts were isolated from human foreskin of a 0-year-old male (HF-0) and from human skin obtained during plastic surgery of a 30-year-old female (HF-30) and a 41-year-old male (HF-41) (Chapter I). Briefly, the cultures were initiated by out-growth from explants of the dermis. The primary cultures were grown in Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical Co., Tokyo) supplemented with 10% fetal bovine serum (FBS, GIBCO Labs., New York), 3.7 mg/ml sodium bicarbonate, 50 IU/ml penicillin (Banyu Pharmaceutical Co., Tokyo) and 50 µg/ml streptomycin (Meiji Seika Kaisha, Tokyo). Subconfluent cells were dispersed with 0.1% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS, pH 7.4) and propagated in the above medium. Cultures at 4th population doubling level (PDL) were frozen and kept in liquid

nitrogen. Skin fibroblasts from 7th PDL to 9th PDL were used throughout the experiments.

Preparation of collagen substrata and cell cultures

A solution of pepsin-treated type I collagen (porcine tendon) in dilute HCl (pH 3.0) at a concentration of 3 mg/ml was obtained from Nitta Gelatin Co. (Osaka). The collagen solution at a concentration of 11 mg/ml was prepared as follows; the above collagen solution (3 mg/ml) was lyophilized and the collagen was redissolved in 0.5M acetic acid with stirring for 3 days at 4 °C to adjust the concentration to 11 mg/ml.

To prepare collagen-coated dishes, 0.67ml of collagen solution (3 mg/ml) was placed onto 35-mm plastic tissue culture dish and dried under a laminar flow of sterile air at 25 °C. Dishes were incubated with 2 ml of culture medium at 37 °C for 2 h prior to use.

Three-dimensional gels of collagen fibrils were prepared in 35-mm plastic tissue culture dishes by rapidly mixing 6 ml of collagen solution (3 mg/ml) with 3 ml of 3x concentrated DMEM with sodium bicarbonate, penicillin and streptomycin, 1 ml of FBS, and 8 ml of DMEM containing 10 % FBS and pipetting 1.5 ml aliquots into the dishes. The collagen-medium solution (1 mg/ml) was incubated at 37 °C for 6 h in a humidified CO_2 incubator before use. Gels at different concentrations of collagen were prepared in a similar manner by using the collagen solution at a concentration of 11 mg/ml. One ml of the cell suspension in DMEM containing 10% FBS ($5x10^4$ cells/ml) was placed on the surface of collagen gels and then incubated at 37 °C in a humidified CO_2 incubator.

For the culture under collagen gels, cells ($5x10^4$ cells) were cultured on 35 mm plastic dishes for 4 h. The medium was removed and 1.5 ml of the above collagen-medium solution (1mg/ml) was overlaid on cell layer. Cells under collagen gel were incubated for 2 h at 37 °C in a humidified CO_2 incubator. Thereafter, 1.5 ml of culture medium without collagen was

overlaid on the surface of gels.

Incorporation of cells within the three-dimensional collagen gels was performed by rapidly mixing a cell suspension with the collagen-medium solution before plating it into the 35 mm plastic tissue culture dishes. The gels containing cells did not always contract uniformly along horizontal axes for 12 days-culture if the gels were not detached from dishes. Thus, peripheral end of gels was scraped at 3 h after starting culture to release the gel from dish. By this procedure, collagen gels were contracted uniformly (Chapter I). The culture medium was renewed every 2 days.

Determination of cell number

The cell number on plastic dishes, on collagen coated dishes, on collagen gels or in collagen gels was determined by measurement of DNA as described by Labarca and Paigen (1980). The amount of DNA was converted to cell number by using the factor of 10 pg DNA/cell. Cells grown on collagen coated dishes and on or in collagen gels were washed 3 times with PBS and incubated with 0.2% bacterial collagenase (Wako Pure Chemical Co., Tokyo)-1mM $CaCl_2$ in PBS for 2 h to dissolve the gels. The floating cells were then collected by centrifugation at 300 g for 10 min. The cell pellets resuspended was combined with the cells which were detached from the dish by treatment with 0.1% trypsin plus 0.02% EDTA in PBS. DNA contents were determined as described above after combined cell suspension was collected by centrifugation. By the above method, almost all the cells were recovered from the gels.

Determination of nutrient diffusion

Two ml of collagen-medium solution (1 mg/ml) was placed in 35 mm plastic tissue culture dishes and incubated at 37 °C for 6 h. The equal volume of culture medium (2 ml) containing 2 μCi of ^{3}H-thymidine (^{3}H-TdR), ^{3}H-proline or ^{125}I-epidermal growth factor (^{125}I-EGF) was overlaid on the surface of gels and the dishes were incubated at 37 °C. After indicated

periods of time, 10 µl of culture medium was collected and counted with a liquid scintillation spectrometer (Aloka, Tokyo) or with a γ-counter (Aloka, Tokyo). ^{3}H-TdR, ^{3}H-proline and ^{125}I-EGF were purchased from New England Nuclear (Boston).

Determination of ^{3}H-thymidine incorporation

Cells on plastic dishes, on collagen coated dishes or on collagen gels were incubated in the culture medium containing 1 µCi of ^{3}H-TdR at 37 °C for 6 h. Cells in collagen gels or in contracted collagen gels incorporated thymidine to a much less amount and so the incubation was continued for 18 h to minimize measurement errors. The isotope incorporation was terminated by transfer of cultures to incubation at 4 °C with a 1000-fold excess amount of unlabeled thymidine. The cultures were washed 3 times with PBS containing 50 µM thymidine. Cells on plastic dishes were treated with 0.1% trypsin-0.02% EDTA-50 µM thymidine in PBS. In case of "on-gel" or "in-gel" culture, cells were treated with 0.2% bacterial collagenase-1 mM $CaCl_2$-50 µM thymidine in PBS and then 0.1% trypsin-0.02% EDTA-50 µM thymidine in PBS. Cell pellets were frozen at -20°C when immediate analysis was not performed. Thawed cells were suspended in 2 ml of 0.1% trypsin-0.02% EDTA in PBS and disrupted with a sonicator. One ml of a sonicated cell suspension was used to determine the DNA contents. One mg of calf thymus DNA was added as a carrier to another 1 ml of cell suspension. The ^{3}H-DNA was precipitated with ice-cold 5% trichloroacetic acid (TCA), collected on glass-fiber filters, washed 4 times with ice-cold 5% TCA , and counted with a liquid scintillation spectrometer (Aloka, Tokyo). The thymidine incorporation was expressed by dpm per µg DNA per hour of incubation.

Determination of cell density in contracted collagen gels

The contracted collagen gels were washed 3 times with PBS and fixed in 10% formaldehyde in PBS pH 7.4. The fixed gels were routinely embedded in paraffin. The sections were stained

with hematoxylin-eosine. Cell density in gels was determined by measuring cell number per unit area of section. Data were calculated by the measurement of 8 sections at each stage.

Electron microscopic observations

Collagen gels were washed 3 times with PBS, minced into a small pieces (ca. 2 mm cube) with a razor blade, and fixed with 4% paraformaldehyde and 5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) for 2 h at 4 °C. The fixed gels were immersed to the above fixative containing 0.25 % tannic acid to enhance the electron density of collagen fibrils for 2 h at 4 °C (Cotta-Pereira et al., 1976). They were washed with 0.1M phosphate buffer (pH 7.4) and post-fixed with 2% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 1 h at 4 °C. They were then dehydrated in graded series of ethanol and embedded in epoxy resin. Ultrathin sections were prepared and stained with uranyl acetate and lead citrate. The sections were examined with an electron microscope. The density of collagen fibrils in gels was determined by the analysis of electron micrographs by using Tospix-II (Toshiba,Tokyo).

3. Results

3.1 Comparison of cell proliferation in "in-gel" culture with "on-gel" culture

The growth curves of human skin fibroblasts (HF-30) on plastic dishes, on the surface of three-dimensional collagen gels ("on-gel" culture), within the three-dimensional collagen gel matrix ("in-gel" culture), and in contracting gel are shown in Fig. III-1. A lag period prior to a logarithmic phase growth of fibroblast in vitro was always found on or within collagen substrata as well as on plastic dishes. The data for a time of lag period and a population doubling time

on various substrata were shown in Table III-1. Fibroblasts cultured on any states of collagen gel substrata, except collagen coated dishes which did not form collagen fibrils (data not shown), indicated a prolonged lag period as compared with cells on plastic dishes (from 1 day to 2 days). Number of fibroblasts in logarithmic scale increased linearly with time. The slopes depended on whether "on-gel" culture or "in-gel" culture. The doubling time of fibroblasts cultured "on-gel" was approximately the same to that on plastic dishes, even if the concentration of collagen was increased from 1 mg/ml to 6.6 mg/ml. A significant repression of proliferation rate of fibroblasts in logarithmic phase growth was observed in "in-gel" culture at a collagen concentration of 1 mg/ml. The repression in growth rate of fibroblasts was found only in culture within the three-dimensional collagen gels, but not when cultured on collagen coated dishes and on or under the surface of collagen gels. Cells under collagen gel were virtually identical with the cells cultured on plastic dish, indicating that the cellular growth was influenced by the substratum to which cells initially attached, but not by the surface which became available after initial attachments had been formed.

The repression of growth rate in logarithmic phase growth by "in-gel" culture was also examined by using other human fibroblasts (HF-0 and HF-41) and under different FBS concentration in a range of 1 % to 10 % (Table III-2). The rate of cell proliferation was not affected by "on-gel" culture as compared with the culture on plastic dishes, while the significant repression was observed in "in-gel" culture.

3.2 Diffusion of nutrients from culture medium into collagen gel

Diffusion of ^{3}H-TdR, ^{3}H-proline or ^{125}I-EGF from culture medium into collagen gel at a concentration of 1 mg/ml was examined in order to estimate diffusion of nutrients into the

gel. Level of ^{3}H-TdR, ^{3}H-proline or ^{125}I-EGF in medium decreased by diffusion as shown in Fig. III-2. Within 20 min after incubation at 37 °C for ^{3}H-TdR and ^{3}H-proline or within 80 min for ^{125}I-EGF, the concentration of the label in medium reached plateau in that the original concentration was reduced to half in equilibrium between medium and the gel which have the equal volumes.

3.3 Relation between reduction in growth rate and contracted gel size

Different number of cells was initially mixed with collagen solution and cultured to obtain various sizes of contracted gels. The collagen gel was more rapidly contracted with increasing the initial number of cells as previously reported. The size of contracted collagen gel (area of gel) and the number of cells in the gel on day 7 were shown in Table III-3. The cell density in contracted gels determined by the measurment of cell number on paraffin sections was also shown in Table III-3. The cell density in contracted gels per cross-sectional area was much lower than that of confluent culture on plastic dish, even if the initial number of cells incorporated in collagen gels was increased. From the actual analysis of the photograph, cells in contracted gels were generally well separated as shown in Fig. III-3A.

The incorporation of tritiated thymidine (^{3}H-TdR) into the trichloroacetic acid (TCA) precipitable fraction of the cell lysate was taken as a measure of mitotic activity or DNA synthesis of cells in logarithmic phase growth (Table III-3). No difference was observed in the rates of DNA synthesis among cells grown on plastic dishes, on collagen coated dishes, or on the surface of collagen gels. The incorporation of ^{3}H-TdR by cells grown within three-dimensional collagen gels was less than a half of that by cells on plastic dishes or on the surface of collagen gels. A further intense repression of DNA synthesis was observed in contracting collagen gel culture.

The extent of ^{3}H-TdR incorporation was repressed with the degree of contraction in collagen gel size. These results indicate that the growth repression in three-dimensional collagen gel was closely correlated to a high density of cells or collagen fibrils, or cell-collagen interactions due to the collagen gel contraction.

3.4 Density of collagen fibrils in contracted collagen gels

The collagen fibrils in contracted collagen gels after 14 days culture was observed with an electron microscope (Fig. III-3B). The collagen fibrils showed typical banding pattern with a 65-70 nm periodicity (Fig. III-3C) and an average diameter of fibrils was 41 ± 24 nm (n=250) which was not different from that of the fibrils formed without cells. The density of collagen fibrils per cross-sectional area in contracted gels was determined by the analysis of electron micrographs as shown in Fig. III-3B. The relation between densities of fibrils and sizes of collagen gels is shown in Fig. III-3D. Data were obtained for cultures on day 0, 2, 7 and 14 after start of gel culture under the condition as follows; initial number of cells was 5×10^{4} cells/gel, initial amount of collagen was 2 mg/gel, and initial gel volume was 2 cm^{3}. The density of fibrils per cross-sectional area in collagen gels was found to be in a reciprocal relation to the collagen gel size which was indicated by the area of collagen gel.

4. Discussion

The fibroblasts surrounded by collagen fibrils in the three-dimensional structure ("in-gel" culture) were repressed in the proliferation rate in logarithmic phase of growth, while the cells cultured on the two-dimensional surface of

collagen gels ("on-gel" culture) were not (Fig. III-1, Table III-1, 2 and 3). Cell proliferation with contracted collagen gel is most markedly repressed (Fig. III-1 and Table III-3). Other human fibroblasts (HF-0 and HF-41) examined have also shown similar results (Table III-2). The slower growth of fibroblasts by "in-gel" culture in logarithmic phase growth may have resulted from the following possible causes.

First, the collagen gels may sterically interfere with free diffusion of nutrients, growth factors, or gas such as O_2 or CO_2 to cells. Diffusion of ^{3}H-TdR or ^{3}H-proline from culture medium into collagen gel at a concentration of 1 mg/ml reached equilibrium within 20 min after incubation at 37°C and that of ^{125}I-EGF reached equilibrium within 80 min (Fig. III-2). It has been also reported that the diffusion of ^{3}H-TdR in contracted collagen gel was not limiting the incorporation into cell (Sarber et al. 1981). As shown in Table I, cell growth under collagen gel was similar to that on plastic dishes, indicating the presence of the collagen fibrils on top half of the cells alone did not retard growth. Namely, overlying collagen matrix which should have interfered with diffusion of nutrients and growth factors as much as, if not more, in case of "in-gel" culture did not appear to cause repression of cell growth rate. Hence, slowered diffusion of nutrients, growth factors, or gas by steric hindrance in gel is unlikely to have a major responsibility for the repression of cell growth rate during the logarithmic growth phase in three-dimensional collagen gels.

Second, high cell density approaching direct cell-to-cell contacts may limit proliferation. Table III indicates number of cells per unit area of cross-section. The highest cell density in gels on day 7 is 171 cells/mm^2, which is much less than that on plastic dishes at confluency (850 cells/mm^2). The more sparse cell density in contracted gel can be confirmed in another way, by comparing the average volume in gel occupied by one cell, 3.6×10^4 μm^3/cell, with the average cell volume, 1.5×10^3 μm^3. Thus, most cells in gels were well separated,

indicating that some factor other than cell-to-cell contact are responsble for the repression of fibroblast-proliferation in contracted gels.

Third, number and distribution of cell-collagen interaction sites per cell affects cytoskeletal architecture (Chapter II) which may eventually result in changing cellular proliferation. It should be noted that cells in the gels are embedded in a homogeneous three-dimensional matrix, while cells on the surface of gels, on collagen coated dishes, and on plastic dishes are grown on a two-dimensional surface. Cells on a two-dimensional surface attached to substrata only by the lower side of cells, and the upper side of cells was free from the substrata as shown in Fig. II-1-1a in ChapterII. Cells in a three-dimensional matrix attached to collagen fibrils equally by all sides of cell membrane (Fig. II-1-1b and Fig II-1-2 in Chapter II). The resultant difference in cellular proliferation may be pertinent to distribution and/or concentration of cell membrane interactions with collagen matrix.

From the study in this chapter, it has been demonstrated that fibroblast growth was suppressed by the interactions with collagen fibrils and the major factor for the growth repression is proposed to be the distribution and/or number of binding sites on cell surface (cell surface receptors) directly with collagen fibrils or indirectly with collagen via fibronectin. Furthermore, it has been reported that these cell surface receptors, called integrin family, link the cytoskeleton with the extracellular matrix (Burridge et al., 1988; Carter and Wayner, 1988; Akiyama et al., 1990). We found in Chapters I and II that the morphology and cytoskeletal organization of fibroblasts induced by the interactions with the three-dimensional network of collagen fibrils were distinct from those observed in the two-dimensional culture on surface of plastic dish or collagen gel. From these evidences, the growth repression induced by the culture within collagen gel is thought to be due to the

intact linkage of collagen fibrils-collagen receptors-cytoskeletons (and/or another linkage of fibronectin bound to collagen fibrils-fibronectin receptor-actin microfilaments). This hypothesis is also supported by the following facts. First, in the culture of fibroblasts on plastic dishes, focal contacts and other sites of cell adhesion are partially disrupted and the associated actin filaments detach from plasma membrane and are disorganized, when the cells were stimulated in growth by the growth factors (Alberts et al., eds., 1989-a). It is suggested that growth stimulation is closely correlated with the disorganization of cytoskeleton. Second, growth of human fibrosarcoma HT1080 cells was not repressed even within collagen gel (Nishiyama et al., in contribution). However, HT1080 cells within collagen gel showed attachment to collagen fibrils and elongate shape similar to normal fibroblasts. On the contrary, actin filaments of HT1080 cells within collagen gel were disorganized, suggesting that no growth inhibition might be due to the disruption of actin filaments.

To confirm the hypothesis, we have examined the preliminary experiments whether fibroblasts growth-arrested in contracted collagen gel are stimulated in proliferation by the disruption of intact linkage from extracellular collagen to intracellular cytoskeleton. In the first experiment, fibroblasts in contracted collagen gel were treated with a low dose of cytochalasin D (0.1 μM) which disrupt actin filaments but does not affect the cell growth on plastic dishes. After 8 days culture with CD and 10% FBS, cell number of fibroblasts in contracted collagen gel was slightly increased by less than 7% as compared to the control culture without CD. Secondly, fibroblasts in contracted collagen gel were treated with 0.1% trypsin or with collagenase (0.03-1.0 unit/ml; collagenase Form III, Advance Biofactures Co., NY) to cleave the binding sites between collagen fibrils and cell surface receptors. In these experiments, we have not yet observed any stimulative effect on cell growth. Thirdly, fibroblasts in contracted

collagen gel were treated with epidermal growth factor (EGF), one of potent mitogens. It has been reported that several mitogenic growth factors stimulates membrane ruffling and disassembly of stress fibres as well as focal adhesions containing fibronectin, its receptor (integrin), and cytoskeletal components, when human epidermoid carcinoma line A431 or Balb/c 3T3 cells were cultured on plastic dishes (Schlessinger and Geiger, 1981; Herman and Pledger, 1985; Herman et al., 1986; Alberts et al., eds., 1989-a). We have expected that a potent growth factor might be able to release the inhibition of growth in contracted collagen gel. However, EGF did not stimulate the mitogenic response of fibroblasts. From these preliminary studies, we did not confirm the above hypothesis, but the quiescent state of fibroblasts in contracted collagen gel is intriguing, in that a potent mitogen, which is well-known to stimulate the growth of fibroblasts in monolayer culture on plastic dishes, is completely inactive for fibroblast proliferation.

High collagen concentration can account for differential growth repression of the cells in the contracted gels. Collagen concentration in the contracted gel was estimated to be 6-15 %, comparable with the in vivo collagen concentration reported previously. The estimation was obtained by two different ways. First, density of collagen fibrils at a plane of cross-section was found to be 28 ± 5 % from the analysis of electron micrographs as shown in Fig. 3B. If density per volume is approximated by (density at plane)$^{3/2}$, this would be calculated to be about 15 % [$(0.28)^{3/2}$= 0.15]. Second, since at least 1.2 mg of collagen remained in a contracted gel with a volume of about 18 mm^3, the concentration is taken to be 6-7 %. Growth of fibroblasts was repressed in dermis where according to Harkness et al. (1958), the concentration of collagen was about 7% of the wet weight in mice. In the case of human dermis, 20% of the wet weight (Bartley et al., 1968) or 80-200 $\mu g/mm^3$ with different ages (Shuster et al., 1975) were reported.

Three-dimensional collagen matrix, but not the two-dimensional collagen matrix, repressed growth rate of fibroblasts. For this distinct observation, the separation of logarithmic phase growth from a lag period prior to logarithmic growth is essential. A prolonged lag period in growth curve of fibroblasts either "on-gel" or "in-gel" was marked in comparison with plastic culture. The prolongation may be due to strong adhesion of cells with collagen fibrils. We presume that lag time failed to be observed by Schor (1980) in the culture of cells on the surface of gels was hidden in an interval of measuring cell numbers (every two or three days), since a lag time was less than 2 days as shown in Fig. III-1. Elsdale and Bard (1972) and Yoshizato et al. (1984 and 1985) have described that by 48 hours after initiating culture, ^{3}H-TdR incorporation in fibroblasts was reduced either on collagen gels or on collagen-coated dishes. They did not report data on incorporation rates at some later stages of growth curve. Their observations for reduced thymidine incorporation by collagen fibrils at an early stage must have included in a large part reduced incorporation due to a prolonged lag period. The initial repression in thymidine incorporation after starting the culture is thus not necessarily reflecting repression in growth rate of logarithmic phase.

Table III-1. Comparison of lag time and population doubling time of human skin fibroblasts (HF-30) cultured with collagen substrata.

Substratum		Lag time(h)	Doubling time(h)
on Plastic dish [a]		24	27 ± 1
on Collagen coated dish		24	28 ± 2
under Collagen gel	(1.0mg/ml)	24	28 ± 2
on Collagen gel	(1.0mg/ml) [a]	48	29 ± 2
	(3.0mg/ml)	48	31 ± 1
	(6.6mg/ml)	48	30 ± 2
in Collagen gel	(1.0mg/ml) [a]	48	42 ± 1

a). Calculated from data shown in Fig. III-1.
Cells ($5x10^4$) were cultured on each collagen substratum. Media containing 10% FBS were renewed every 2 days. Data were calculated from the growth curves by the measurement of 3 dishes.

Table III-2. Repression of growth rate by "in-gel" culture

Human fibroblasts	FBS (%)	Doubling time (h) on Plastic	on Collagen gel	in Collagen gel
HF-0 (15th PDL)	10	27 ± 1	29 ± 1	48 ± 1
(32nd PDL)	10	75 ± 4	76 ± 5	120 ±10
HF-30 (8th PDL)	1	59 ±11	62 ± 7	123 ±17
	2	43 ± 7	47 ± 5	78 ± 9
	5	31 ± 3	34 ± 4	63 ± 5
	10[a]	27 ± 1	29 ± 2	42 ± 1
HF-41 (8th PDL)	10	50 ± 3	48 ± 4	91 ± 5

a). Calculated from data shown in Fig. III-1.
Cells ($5x10^4$) were cultured on each collagen substratum (1.0 mg/ml). Media containing FBS were renewed every 2 days. Data were calculated from the growth curves by the measurement of 3 dishes.

Table III-3. ^{3}H-Thymidine incorporation into human skin fibroblasts (HF-30) in logarithmic phase growth

Substratum	Initial cell number (10^4cells)	Days in culture	Number of cells (10^4cells)	Cell density (cells/mm^2)	Collagen gel size (mm^2)	^{3}H-TdR in DNA[b] (10^3dpm/μgDNA/h)
on Plastic dish	5	3	26±1	290±10[a]	--	30 ± 2
on Collagen coated dish	5	3	24±2	267±22	--	28 ± 3
on Collagen gel	5	3	17±2	213±25	900	29 ± 3
in Collagen gel	5	4	12±1	10± 2	900	14 ± 0.2
in Contracted collagen gel	5	7	12±0	41± 5	71±1	3.0±0.1
	5	14	24±1	70± 6	40±5	1.0±0.2
	10	7	19±1	62± 4	50±3	1.4±0.1
	20	7	25±1	121±13	40±2	1.3±0.1
	34	7	34±1	171±10	30±1	0.9±0.1

a). Cell density on plastic dish at confluency was 850±7 cells/mm^2.
b). Data represent the triplicate determinations ± SD.

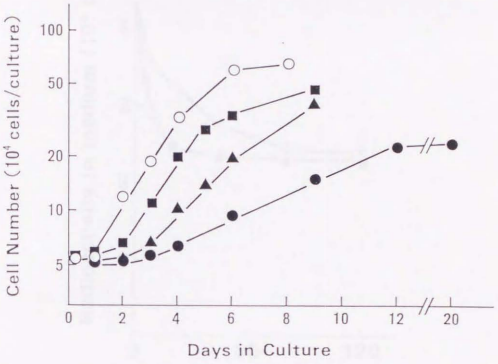

Fig. III-1. Effect of different substrata on growth of human skin fibroblasts. Human skin fibrobalsts (HF-30) in DMEM containing 10% FBS were plated at an intial density of $5x10^4$ cells/dish and cell number determined at indicated times as described in Materials and Methods. Each point is the mean of triplicate determinations. The standard deviation was in all points less than 10%. ○ cells on plastic dishes; ■ cells on the surface of collagen gel; ▲ cells within the collagen gel; ● cells in the contracting collagen gel.

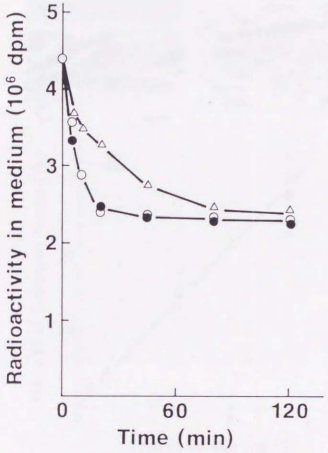

Fig. III-2. Diffusion of ^{3}H-TdR, ^{3}H-proline or ^{125}I-EGF into collagen gel. Collagen gels (1 mg/ml) were prepared in 35 mm plastic tissue culture dishes. Diffusion of the label (originally 4.4 x 10^6 dpm) from culture medium into collagen gel was measuresd. Each points is the mean of triplicate determinations. The standard deviation was in all points less than 5 %. ● ^{3}H-TdR; ○ ^{3}H-proline; △ ^{125}I-EGF.

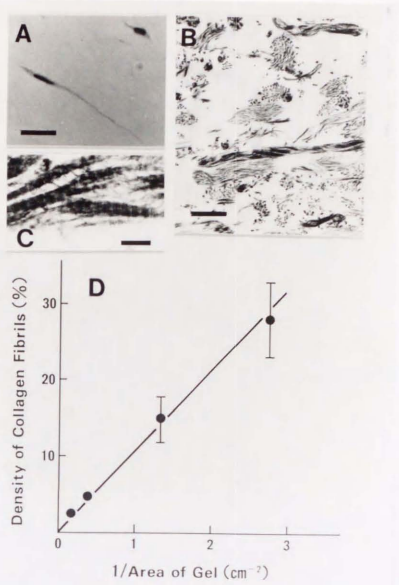

Fig. III-3. (A). A micrograph of paraffin-embedded section of contracted collagen gel with human skin fibroblasts (HF-30) after 14 days culture. The bar corresponds to 25 μm.
(B). An electron micrograph of contracted collagen gel with human skin fibroblasts (HF-30) after 14 days culture. The bar corresponds to 1 μm.
(C). An electron micrograph of collagen fibrils under the condition of (B). The bar corresponds to 200 nm.
(D). Relationship between the density of collagen fibrils per cross-sectional area in gels and the size of contracted collagen gels.
(Electron microscopy was examined by Dr. E. Adachi in Osaka University Medical School.)

Chapter IV

Characteristics of the Quiescent State of Human Dermal Fibroblasts in Contracted Collagen Gel

Part A : No response to epidermal growth factor and its stimulant.

Part B : Growth response to other growth factors.

Part A : No response to epidermal growth factor and its stimulant.

[Summary]

The study in Chapter III indicated that human fibroblasts in the contracted collagen gel did not proliferate in the presence of 10 % serum even though there was no apparent cell-cell contact. We interpreted this cell growth inhibition as being caused by a high level of cell-collagen fibril interactions or cell-matrix contact inhibition. In the present study, the effect of epidermal growth factor (EGF) on fibroblast proliferation in the contracted collagen gel was examined to elucidate the mechanism of repressive effect of cell growth by reconstituted collagen fibrils. Non-dividing cells at confluency on a plastic dish or on collagen gel responded to the added EGF and multiplied, while the cells in the contracted collagen gel did not show any growth response to EGF at concentrations up to 100 ng/ml. Binding assay of ^{125}I-EGF to the cells showed that the number of binding sites and the binding constant obtained from Scatchard analysis were essentially unchanged in the contracted collagen gel, indicating that EGF receptors were not masked by collagen fibrils but that the intracellular events including signal transduction after binding of EGF were blocked. The blocking was suppressed by the addition of saikosaponin b_1. These results suggested that the quiescent fibroblasts in the contracted collagen gel were in a distinct state from previously known quiescent states of cultured cells, namely quiescent states due to cell-cell contact inhibition at confluency or to deficiency of growth factors. The mechanism of the effect of saikosaponin b_1, which has a potent saponin activity, is discussed.

1. Introduction

A repressive effect of the three-dimensional collagen matrix on fibroblast proliferation, in contrast to the effect of the two-dimensional matrix, was reported in Chapter III. A collagen gel containing fibroblasts contracts spontaneously when the end of the gel is detached from the dish as shown in Chapter I. The repression of cell growth was more marked in a contracted collagen gel than in an uncontracted gel still attached to the dish and finally fibroblasts in a contracted collagen gel stopped proliferation in the presence of sufficient serum without apparent cell-to-cell contacts. We concluded that the high density of collagen fibrils in the contracted collagen gels, resulting in a high level of cell-matrix interaction, was the major reason for the growth repression. Compared to quiescent cells at confluency or in cell-cell contact inhibition, the state may be called cell-matrix contact inhibition, which is thought to be mediated through the interactions of cell surface receptors with collagen fibrils.

Epidermal growth factor (EGF), a soluble peptide factor, regulates various cellular activities such as proliferation, differentiation and metabolic activity (Antoniades and Owen, 1982; Carpenter and Cohen, 1984; Colige et al., 1988; Hata et al. 1988, Kawamoto et al., 1989). Various cells of epithelial or mesenchymal origin respond to EGF when grown as monolayers *in vitro*. It has been reported that the collagen gel matrix modulated the growth response to EGF of corneal epithelial cells (Gospodarowicz and Tauber, 1980) or that the collagen gel caused a decrease in the effect of EGF on collagen synthesis by fibroblasts (Colige et al., 1988). Nakagawa et al. (1989) and Rhudy and McPherson (1988) have shown that the collagen matrix also suppressed ^{3}H-thymidine incorporation stimulated by peptide growth factors. In terms of cell adhesion, a speculative model of growth regulation is proposed when a cell cultured on plastic is stimulated to proliferate

by growth factors (Burridge et al., 1988; Alberts et al., eds., 1989-a). Binding of growth factor to its receptor leads to phosphorylation of the c-src protein. This protein kinase is activated and in turn phosphorylates tyrosine residues on neighboring transmembrane cell-adhesion proteins, including the fibronectin receptor. As a results, focal contacts and other sites of cell adhesion are partially disrupted and the associated actin filaments detach temporarily from the plasma membrane. Growth factors seem to act, in part, by transiently weakening the adhesions on which normal cell proliferation depends. Growth regulation by growth factors is closely coupled with the organization of cell adhesion.

In this chapter, the effect of reconstituted type I collagen fibrils on the growth response to human dermal fibroblasts to growth factors was examined to elucidate the mechanism of growth repression by the interactions between a cell (via surface receptors) and collagen fibrils. In Part 1, we found that EGF was able to bind to its receptor of fibroblasts which had apparently stopped dividing in the contracted collagen gel, but the cells did not respond to stimulation with EGF alone. However, they resumed multiplying upon combined addition of EGF and saikosaponin b_1, one of the biologically active or erythrolytic saponins, purified from the root of Bupleurum falcatum L. Sikosaponin b_1 is one of the antagonist to cell-matrix contact inhibition.

2. Materials and Methods

Cell culture

Fibroblasts were isolated from human foreskin of a 0-year-old male (HF-0) and from human skin of a 30-year-old female (HF-30) and a 41-year-old male (HF-41) obtained during plastic surgery, as described in Chapter I. Cultures at the 4th population doubling level (PDL) were frozen and kept in

liquid nitrogen. Human fibroblasts from 7th PDL to 10th PDL were used throughout the experiments.

Preparation of collagen substrata and cell cultures

A solution of pepsin-treated type I collagen (porcine tendon) in dilute HCl (pH 3.0) at a concentration of 3 mg/ml was obtained from Nitta Gelatin Co. (Osaka).

To prepare collagen-coated dishes, 0.67 ml of collagen solution (3 mg/ml) was placed in each 35-mm plastic tissue culture dish, which was then dried under a laminar flow of sterile air at 25 °C. Dishes were incubated with 2 ml of culture medium at 37 °C for 2 h prior to use.

For culture with Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical Co., Tokyo) containing 0.2% fetal bovine serum (FBS, GIBCO Labs., New York) on three-dimensional gels of collagen fibrils in 35-mm plastic tissue culture dishes, 1.5 ml aliquots of a solution (collagen-medium solution) obtained by rapidly mixing 6 ml of collagen solution (3 mg/ml), 3 ml of 3x concentrated DMEM with sodium bicarbonate, penicillin and streptomycin, 0.5 ml of FBS, and 8.5 ml of DMEM containing 5% FBS were pipetted into the dishes. The collagen-medium solution (1 mg/ml) was incubated at 37 °C for 3 h in a humidified CO_2 incubator. One ml of the cell suspension in DMEM containing 5% FBS ($2x10^5$ cells/ml) was placed on the surface of each collagen gel and the plate was incubated for 6 h at 37 °C. The cells were cultured in 0.2% FBS-DMEM for 24 h at 37 °C, after being washed 4 times with 2 ml of 0.2% FBS-DMEM for 30 min at 37 °C.

In the case of culture under collagen gel, cells ($2x10^5$ cells) were placed on a plastic dish and incubated with 5% FBS-DMEM at 37 °C for 6 h. The cultured cells were washed twice with 0.2% FBS-DMEM, and then 1.5 ml of the collagen-medium solution (1 mg/ml, 0.2% FBS-DMEM) was overlaid on the cell layer. Cells under the gel were incubated for 3 h at 37 °C, and then 1.5 ml of 0.2% FBS-DMEM was overlaid on the surface of gel. The incubation was continued for 24 h at 37

°C.

Incorporation of cells within the three-dimensional collagen gel was performed by rapidly mixing a cell suspension with the collagen-medium solution (1 mg/ml of collagen and 5% FBS-DMEM), then plating the mixture into 35-mm plastic tissue culture dishes. The gels containing cells ($2x10^5$ cells/2 ml) were incubated for 6 h at 37 °C and then washed 4 times with 0.2% FBS-DMEM for 30 min at 37 °C. The gels were overlaid with 1.5 ml of 0.2% FBS-DMEM. The incubation was continued for 24 h at 37 °C. The effect of EGF was examined by culturing the cells with 0.2% FBS-DMEM containing EGF (24 ng/ml) for 9-72 h.

Preparation of contracted collagen gels containing fibroblasts

Collagen solution containing fibroblasts was prepared by mixing 6 ml of collagen solution (3 mg/ml) with 3 ml of 3x concentrated DMEM, 1 ml of FBS, 5 ml of 10% FBS-DMEM and 3 ml of the cell suspension in 10% FBS-DMEM ($9x10^5$ cells/ml) at 4 °C to give a final density of $1.5x10^5$ cells/ml and a collagen concentration of 1.0 mg/ml. One ml of the solution containing cells and collagen was placed in a bacteriological 24-well flat-bottomed plastic tray (Terumo Co., Tokyo) and then brought to 37 °C for the gel formation. The edge of each gel was scraped off at 3 h after starting culture to release the gel from the well; this procedure assured uniform collagen gel contraction (Nishiyama et al., 1988). The incubation of the contracted gel was continued at 37 °C for 8 days until cells ceased to multiply. The culture medium was renewed every 2 days. Fibroblasts arrested in the contracted collagen gel were incubated for 2 days with culture medium and then cultured with EGF and/or saikosaponin b_1 for 9-72 h.

Cell culture under a low serum condition or at confluency on plastic dishes

In the case of culture under a low serum condition, cells ($2x10^5$ cells) were placed on a 35-mm plastic tissue culture dish and incubated with 5% FBS-DMEM at 37 °C for 6 h. The

cultured cells were washed twice with 0.2% FBS-DMEM and incubated with 0.2% FBS-DMEM at 37 °C for 24 h. The effect of EGF and/or saikosaponin b_1 was examined by culturing the cells with 0.2% FBS-DMEM containing EGF and/or saikosaponin b_1 for 9-72 h.

In the case of monolayer culture, cells ($5x10^4$ cells) were placed on a 24-well flat-bottomed tissue culture tray and incubated with 10% FBS-DMEM at 37 °C for 4 or 5 days until cells reached at confluency. The culture medium was renewed every 2 days. Fibroblasts arrested at confluency in 10% FBS-DMEM were then cultured with EGF and/or saikosaponin b_1 for 9-72 h.

Determination of cell number

The cell number was determined by measurement of DNA as described by Labarca and Paigen (1980). The amount of DNA was converted to cell number by using the factor of 10 pg DNA/cell. Cells grown on or in collagen gels were placed in a plastic tube and incubated with 0.2% bacterial collagenase (Wako Pure Chemical Co., Tokyo)-1 mM $CaCl_2$ in phosphate-buffered saline (PBS, pH 7.4) for 2 h to dissolve the gels. The floating cells were then collected by centrifugation at 300 g for 10 min. The cell pellets were washed twice with 0.1% trypsin plus 0.02% EDTA in PBS.

^{125}I-EGF binding assay

Mouse EGF and bovine serum albumin (BSA) were obtained from Sigma (St. Louis). ^{125}I-EGF was purchased from New England Nuclear (Boston).

In the case of monolayer culture on plastic dishes, ^{125}I-EGF binding assay was performed at 4 °C using a modification of the reported method (Carpenter and Cohen, 1976). Non-specific binding was measured as the radioactivity bound to cells in a medium containing a 100-fold molar excess of unlabeled EGF. Non-specific binding was always less than 10% of the total.

The effect of EGF was examined on the cells which had ceased to divide in the contracted collagen gels at 8 days. To measure ^{125}I-EGF binding, the contracted collagen gels were washed twice with DMEM and incubated with DMEM containing 0.1% BSA and 25 mM Hepes buffer pH 7.4 (binding medium) for 1 h at 37 °C. The contracted collagen gels were incubated with 200 µl of binding medium containing ^{125}I-EGF at 4 °C for an indicated period of time. To terminate the incubation, the contracted collagen gels were washed 4 times in ice-cold Hank's balanced salt solution (pH 7.4) containing 25 mM Hepes for 10 min at 0 °C. Then, the contracted collagen gels were dissolved in 1 N NaOH. The radioactivity was counted in a γ-counter (Aloka, Tokyo). For non-specific binding the radioactivity was measured with the same procedure in a medium containing a 100-fold molar excess of unlabeled EGF. The non-specific binding in the contracted gel was rather high compared with the case of plastic culture, but was less than 30 % of the total. Binding assays showed experimental errors within ±10 %.

Other materials

Saikosaponins a, b_1, b_2, c and d were purchased from Koshiro Pharmaceutical Co. (Osaka). Platelet-derived growth factor (PDGF) from human platelets, basic fibroblast growth factor (b-FGF) from bovine brain and transforming growth factor β (TGF-β) from porcine platelets were purchased from R & D Systems Inc. (Minneapolis, MN).

3. Results

3.1 Effect of reconstituted type I collagen gel on growth response of fibroblasts to EGF

The growth response to EGF of human dermal fibroblasts (HF-30) in mitotic arrest on plastic dishes, on collagen-

coated dishes, on the surface of three-dimensional collagen gels ("on-gel" culture), under collagen gels ("under-gel" culture) and within collagen gels ("in-gel" culture) is shown in Table IV-1. Fibroblasts cultured with any of the collagen substrata responded to EGF after incubation for 72 h, though the growth response to EGF was retarded in "in-gel" culture compared to other cultures including "on-gel" or "under-gel" culture. The number of cells in "in-gel" culture without contraction of the gel after incubation with EGF for 72 h was increased by 28 ± 2 % as compared to the control culture in the absence of EGF. Thus the collagen matrix itself without contraction essentially did not interfere with the mitotic response of cultured cells to EGF.

3.2 Growth response to EGF of quiescent fibroblasts in the contracted collagen gel

Collagen gels containing fibroblasts and 10% FBS began to contract rapidly when the gel attachment to the dish was released. The human dermal fibroblasts in the contracted gels showed a decreased proliferation rate and finally ceased to divide. The cessation of proliferation of the human dermal fibroblasts in the contracted gel was completed within 8 days after the start of culture under the conditions of the present study. The time course curves of growth response to EGF of fibroblasts arrested at confluency on plastic dishes or in the contracted gels are shown in Fig. IV-1. Quiescent fibroblasts at confluency resumed multiplying after more than 24 h upon exposure to EGF in the concentration range from 0.1 ng/ml to 100 ng/ml, while the cells in the contracted gels did not respond to EGF in the same concentration range (Figs. IV-1 and IV-2).

No response to EGF in the contracted gels was also observed by using other human fibroblasts (HF-0 and HF-41) under the condition with 10% FBS (data not shown).

3.3 Binding of EGF to quiescent fibroblasts in the contracted collagen gel

Time courses of the EGF binding to fibroblasts (HF-30) on plastic dishes and to those in the contracted gels at 4 °C are shown in Fig. IV-3A. The fibroblasts on plastic dishes were tested 2 days after reaching confluency and those in contracted gels were examined 2 days after the cessation of proliferation. The amount of EGF bound with fibroblasts on plastic dishes reached a plateau after incubation for 1.5 h, while in the contracted gels the plateau for the bound EGF was attained after incubation with EGF for 4 h.

A Scatchard plot (Fig. IV-3B) of EGF binding with the fibroblasts on plastic dishes gave a straight line, which could be interpreted in terms of a single class of binding sites with a Kd of 4×10^{-9} M and a maximum binding capacity of 7×10^{4} sites per cell. A similar plot of EGF binding with the fibroblasts in the contracted gels also gave a straight line, which could be interpreted in terms of a single class of binding sites with a Kd of 4×10^{-9} M and a maximum binding capacity of 6×10^{4} sites per cell. Fibroblasts in the contracted gels thus appear to have a slightly smaller number of binding sites/cell than those in monolayer on plastic dishes, while the values of affinity constant of the receptor were identical in the two cases.

3.4 Mitotic effect of a combination of EGF and saikosaponin b_1 on the growth of quiescent fibroblasts in the contracted collagen gel

Saikosaponins are biologically active components (mainly saikosaponins a, b_1, b_2, c and d) from the root of Bupleurum falcatum L. Saikosaponins have been used as drugs for hepatobiliary diseases in oriental traditional medicine. The structural formula of saikosaponin b_1 is shown in Fig. IV-4.

The time course of the growth response of quiescent

fibroblasts (HF-30) in the contracted gels to the combination of EGF (24 ng/ml) and saikosaponin b_1 ($1x10^{-6}$ M) is shown in Fig. 5. Neither EGF alone nor saikosaponin b_1 alone stimulated the growth, while the combination of the two was found to stimulate the proliferation of fibroblasts arrested in the contracted gels. Dose dependency of growth stimulation by the combination of EGF and saikosaponin b_1 was determined after 60 h of incubation at a fixed concentration of EGF (24 ng/ml), and with various concentrations of saikosaponin b_1 from $3x10^{-8}$ M to $3x10^{-5}$ M (Fig. IV-5B). Doses higher than $1x10^{-4}$ M saikosaponin b_1, had a cytotoxic effect. Saikosaponin b_1 alone at a concentration of less than $3x10^{-6}$ M did not stimulate the growth of fibroblasts in the contracted gels. The quiescent cells in the contracted gels recovered proliferative activity upon exposure to saikosaponin b_1 alone in the concentration range from $3x10^{-6}$ M to $3x10^{-5}$ M. The mitotic effect of combination of EGF and saikosaponin b_1 was also observed by using other human fibroblasts (HF-41).

The stimulative effect of saikosaponin b_1 alone or the combination of EGF and saikosaponin b_1 on fibroblast growth was also observed in the culture under a low serum condition or at confluency on plastic dishes (Table IV-2). In addition, saikosaponin b_1 was found to enhance the mitotic response of quiescent fibroblasts in the contracted gels to other growth factors (b-FGF, PDGF and TGF-β) (Table IV-3). Saikosaponin b_1, therefore is a growth factor with an entirely new chemical structure.

4. Discussion

The present study demonstrated that human fibroblasts in contracted collagen gel did not multiply in response to EGF alone, but that they multiplied in response to the combination of EGF and saikosaponin b_1. Since EGF is known and was

confirmed here (Table IV-1, Figs. IV-1 and IV-2) to be a potent mitogen for quiescent fibroblasts at confluency or at serum depletion, the negative response to EGF is a distinct characteristic of the cells in contracted collagen gel, where the density of cell-collagen interaction sites may be as high as that in vivo (Figs. IV-1 and IV-2). Quiescent states of normal epithelial cells or fibroblasts plated on a dish are provided by two conditions (Alberts et al.,eds., 1989-a). First, normal cell proliferation in culture generally stopped multipling under the depletion of serum containing growth factors. Second, normal cells plated on a dish in the presence of serum will adhere to the surface and spread out, and divide until a confluent monolayer is formed in which neighboring cells touch one another. At this point normal cells stop dividing (a phenomenon known as density-dependent inhibition of cell division, but a molecular mechanism still unkown), called cell-cell contact inhibition. Both quiescent states are known to be released by the potent mitogen such as growth factors. Thus the contracted collagen gel provides another quiescent state for human fibroblasts. In Chapter III, the repressed growth of fibroblasts in contracted collagen gel was ascribed to the high density of collagen-cell interaction sites, or cell-matrix contact inhibition. Since EGF binding to the cells was essentially unchanged (Fig. IV-3), and the combination of EGF and saikosaponin b_1 stimulated cell growth (Fig. IV-5), the negative response to EGF of the fibroblasts in the contracted collagen gel may be due to a block in the signal transduction pathway of EGF and other intracellular events induced by EGF, caused by the strong interaction between the cell and collagen fibrils.

A growth signaling pathway leading to cell division in general consists of several reaction steps; 1) binding of an extracellular signal such as a growth factor with the specific receptor on the cell membranes, 2) rapid internalization and degradation of growth factor which binds to cell surface receptors by receptor-mediated endocytosis, and 3) signal

conversion to intracellular events after the binding and/or internalization, ultimately transmitted to the nucleus (Antoniades and Owen, 1982; Carpenter and Cohen, 1984; Rozengurt, 1986). One of intracellular events by the signal transduction after binding of growth factors is known that the cell's focal contacts on plastic, where the cell is bound tightly to substrata by a cell-matrix junction involving the fibronectin receptor (and other transmembrane adhesion molecules in the integrin family) and actin filaments (and other cytoskeleton) on its intracellular side, change their structure: focal contacts and other sites of cell adhesion are partially disappeared and the actin filaments that were anchored there are temporarily disrupted (Burridge et al., 1988; Alberts et al., eds., 1989-a). Thus, in stimulating a quiescent cell on plastic dishes to divide, the treatment of growth factors appears to loosen cell adhesions. Several growth factors, EGF, FGF, PDGF, TGF-β, and cholera toxin, are known to have these activity (Burridge et al., 1988).

Direct binding of extracellular collagen fibrils with the cell surface may affect not only the extracellular signaling pathway, but also the later intracellular events. The present study (Fig. IV-3) indicated that EGF binding with the cells was not affected by the collagen fibrils except for the reaction rate of binding; a longer time was required to bind, though it was much shorter than would be expected from a comparison of the times required for cell growth in total (Fig. IV-3A). An alternative interpretation of the cell-matrix contact inhibition is that general cellular activity including the growth signal transduction might be repressed through interference by the collagen fibrils. However, the present finding does not favor this interpretation. That is, saikosaponin b_1 at a concentration below 10^{-6} M (added with EGF) restores the mitogenic activity of EGF toward the cells. It was also found in Part 2 that PDGF stimulates the proliferation of fibroblasts growth-arrested by the cell-matrix contact inhibition.

From the findings that the two-dimensional collagen gel surface affected the cell growth or the growth stimulation by EGF as much as the plastic dish and that the three-dimensional collagen matrix without contraction did not completely repress the cell proliferation or the response to EGF (Table IV-1), it seems likely that the number of cell-collagen interaction sites per cell and their distribution are important for the regulation of cellular growth signal transduction and other intracellular events as loosening cell-collagen adhesions (or interactions). In the previous reports, morphological and biosynthetic changes in fibroblasts induced by the interactions with the three-dimensional network of collagen fibrils were distinct from those observed in the two-dimensional culture on the surface of a plastic dish or collagen gel (Chapter I and II; Tomasek and Hay, 1984; Nusgens et al., 1984; Colige et al., 1988; Tsunenaga et al., 1989). We have suggested that the repression of cell growth by collagen fibrils in the three-dimensional gels is not due to direct cell-cell contact, but instead is dependent on the distribution and number of contact sites between a cell and collagen fibrils (Chapter III). It was also confirmed in the present study that the interaction between the whole cell surface and the dense three-dimensional network of collagen fibrils was most effective in repressing the cell proliferation.

Above results demonstrate the characteristics of a quiescent state of fibroblasts in contracted collagen gel, presumably due to the large number of contact sites on the whole cell surface. The state can be called cell-matrix contact inhibition; both inhibition of intracellular signal transduction pathway by EGF and inhibition of transient disorganization of cell adhesion sites (or motility).

Saikosaponins are known to have a hemolytic activity and to cause a change in the fluidity of erythrocyte membranes (Abe et al., 1978 and 1980). The major components of saikosaponins are saikosaponins a, b_1, b_2, c and d. The

effect of each on the plasma membrane is different from that of the others. However, the interaction or reaction of saikosaponins with biological membranes of human nucleate cells such as fibroblasts has rarely been reported. We found in the present study that saikosaponin b_1 facilitated the recovery of mitotic response to EGF of the quiescent fibroblasts in the contracted gels (Fig. IV-5) as well as that of cells growth-arrested under a low serum condition and at confluency on plastic dishes (Table IV-2). The stimulative effect of saikosaponin b_1 on the mitotic response to b-FGF, PDGF and TGF-β in the contracted gels was also demonstrated in Table IV-3. As shown in Fig. IV-5B, saikosaponin b_1 alone stimulated the growth of fibroblasts in the contracted gels in the concentration range from $3x10^{-6}$ M to $3x10^{-5}$ M. This stimulation may be due to its combined effect with growth factors contained in 10 % serum. Saikosaponin b_1 might have the potential to reactivate the EGF stimulation inhibited by cell-matrix contact inhibition as following: 1. a release of interference of intracellular signal transduction by EGF; 2. a weakening of cell-collagen fibrils adhesion; 3. an activation of alternative pathway of signal transduction. The finding could provide a clue to the location of the blocking site of growth stimulation by growth factors.

Saikosaponins a, c and d had no activity to potentiate the mitotic response, while saikosaponin b_2, which has the same structure as saikosaponin b_1 (Fig. IV-4) except for the position of 16-OH, was also effective in reactivating the mitotic response to EGF in the contracted gels (unpublished data). Further investigation is required to clarify the action of saikosaponin b_1 on the regulation of the growth response to EGF of quiescent fibroblasts in contracted collagen gels.

Table IV-1. Comparison of growth response to EGF of human dermal fibroblasts (HF-30) cultured with collagen substrata.

Substratum	Growth Stimulation (% of control)
on plastic dish	142 ± 1
on collagen-coated dish	143 ± 4
under collagen gel	140 ± 4
on collagen gel	138 ± 2
in collagen gel	128 ± 2

Cells (2.0×10^5) in mitotic arrest in culture under a low serum condition (0.2% FBS) were incubated with 24 ng/ml of EGF. After incubation for 72 h, the cell number was determined as described in Materials and Methods. The data were expressed as % cell number with respect to the controlculture in the medium without EGF and values are the mean of triplicate determinations ± SD.

Table IV-2. Growth stimulation by the combination of EGF and saikosaponin b_1 in the culture under a low serum condition or at confluency on plastic dishes

Concentration of Saikosaponin b_1 (M)	Growth Stimulation (% of control) under a low serum		at confluency	
	-	EGF	-	EGF
0	100 ± 2	133 ± 2	100 ± 2	129 ± 3
10^{-7}	100 ± 4	139 ± 2	104 ± 2	128 ± 1
10^{-6}	111 ± 2	154 ± 2	108 ± 1	137 ± 3
10^{-5}	115 ± 2	166 ± 4	112 ± 2	141 ± 1

Fibroblasts (HF-30) growth-arrested in culture under a low serum condition (0.2% FBS) or at confluency (10% FBS) on plastic dishes were incubated with saikosaponin b_1 or the combination of EGF (24 ng/ml) and saikosaponin b_1. After incubation for 48 h, the cell number was determined as described in Materials and Methods. The data are expressed as % cell number with respect to the control culture, 2.0×10^5 cells for a low serum condition and 2.4×10^5 cells for a confluent culture, in the medium without EGF and saikosaponin b_1. Values are the mean of triplicate determinations ± SD.

Table IV-3. Growth stimulation in contracted collagen gel by the combination of growth factor and saikosaponin b_1

Growth Factor	Growth Stimulation (% of control)	
	-	saikosaponin b_1
EGF	101 ± 3	121 ± 2
b-FGF	105 ± 2	123 ± 2
PDGF	125 ± 3	141 ± 4
TGF-β	101 ± 2	111 ± 1

Fibroblasts (HF-30) growth-arrested in contracted collagen gels were treated with a growth factor or combinations of growth factor and saikosaponin b_1 (10^{-6} M). After incubation for 60 h, the cell number was determined as described in Materials and Methods. The data are expressed as % cell number with respect to the control culture, 2.1×10^5 cells, without growth factors and saikosaponin b_1. Values are the mean of triplicate determinations ± SD. Concentration of growth fator: EGF 24 ng/ml; b-FGF 10 ng/ml; PDGF 30 ng/ml; TGF-β 10 ng/ml.

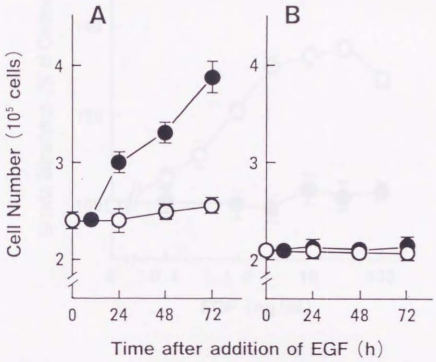

Fig.IV-1. Time course of growth response to EGF of human dermal fibroblasts in contracted collagen gel. Fibroblasts (HF-30) in mitotic arrest in culture with 10% FBS at confluency (A) and in contracted collagen gel (B) were incubated with 24 ng/ml of EGF. The medium was renewed at 48 h. After incubation for the times indicated, the cell number was determined as described in Materials and Methods. Each point represents the mean of triplicate determinations ± SD. ○ : control culture; ● : EGF.

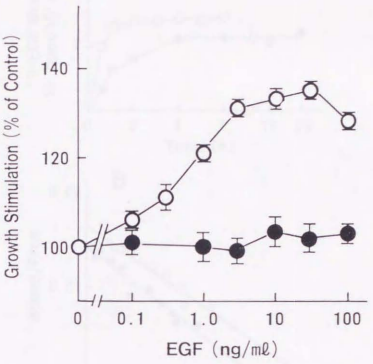

Fig.IV-2. Dose-response relationship for proliferative effect of EGF on human dermal fibroblasts in contracted collagen gel. Fibroblasts (HF-30) were grown on plastic dishes (○) or in contracted gel (●) in 10% FBS-DMEM. Cultures were used 2 days after the cessation of proliferation. At this stage, EGF at the concentrations indicated was added to 10% FBS-DMEM. After incubation for 60 h, the cell number was determined as described in Materials and Methods. The ordinate, growth stimulation, is expressed as % cell number, cell numbers at 60 h incubation with various concentration of EGF vs. those, 2.3×10^5 cells for monolayer culture and 2.1×10^5 cells for contracted gel culture, at 60 h incubation without EGF (control). Each point represents the mean of triplicate determinations ± SD.

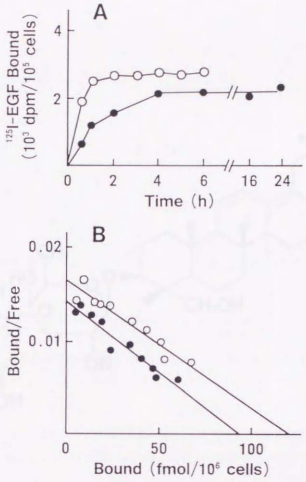

Fig.IV-3. EGF binding to human dermal fibroblasts in contracted collagen gel. Fibroblasts (HF-30) were grown on plastic dishes or in contracted collagen gels in 10% FBS-DMEM. Cultures were used 2 days after the cessation of proliferation. EGF binding was determined at 4 °C as described in Materials and Methods. The data represent the specific (total minus non specific) binding and values are the mean of triplicate determinations. A: time course of EGF binding; B: Scatchard plots of EGF binding data. ○ : cells on plastic dishes; ● : cells in contracted collagen gels.

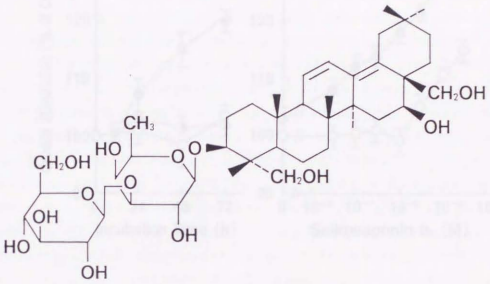

Fig.IV-4. Structural formula of saikosaponin b_1.

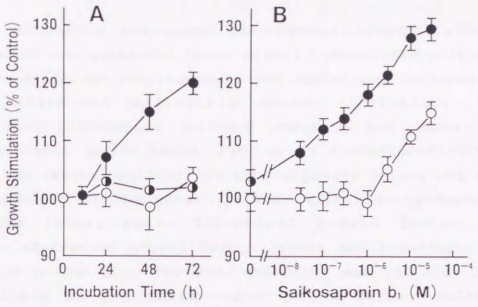

Fig.IV-5. Growth stimulation in contracted collagen gel by the combination of EGF and saikosaponin b_1. Human dermal fibroblasts (HF-30) in contracted gel were cultured in 10 % FBS-DMEM. Cultures were used 2 days after the cessation of proliferation. At this stage, EGF, saikosaponin b_1 or the combination of both was added to 10% FBS-DMEM. A: time course curves of growth response to 24 ng/ml of EGF (◑), $1x10^{-6}$ M saikosaponin b_1 (○) or the combination of EGF (24 ng/ml) and saikosaponin b_1 ($1x10^{-6}$ M) (●). B: dose-dependency of growth response to saikosaponin b_1 (○) or saikosaponin b_1 with EGF (24 ng/ml) (●) after incubation for 60 h. The cell number was determined as described in Materials and Methods. The data were expressed as % cell number with respect to the control culture, $2.2x10^5$ cells, in the medium without EGF and saikosaponin b_1. Each point represents the mean of triplicate determinations ± SD.

Part 2 : Growth response to other growth factors.

[Summary]

Mitogenic responses to various growth factors were compared for quiescent human dermal fibroblasts cultured under three different conditions; serum depletion, cell-cell contact inhibition and cell-matrix contact inhibition. The non-dividing fibroblasts cultured under a low serum condition (0.2% fetal bovine serum, FBS) or in a confluent culture with 10% FBS resumed multiplying upon exposure to any one of or any combination of the growth factors examined; epidermal growth factor (EGF), basic fibroblast growth factor (b-FGF), platelet-derived growth factor (PDGF) and transforming growth factor β (TGF-β). The only exception was the lack of effect of TGF-β on the cells under a low serum condition. In contrast, the proliferation of fibroblasts which were growth-arrested in contracted collagen gel by cell-matrix contact inhibition was not stimulated by any of the growth factors examined except for PDGF. It is currently accepted that the mechanism of growth stimulation or signal transduction after binding of each growth factor to the specific receptor depends on the kind of growth factor. The results suggest that the signal transductions delivered by EGF, b-FGF or TGF-β are inactivated by a high level of interaction of collagen fibrils with the cell membrane (under the condition of cell-matrix contact inhibition), whereas the signal transduction by PDGF is unaffected. The finding also supports the existence of a specific growth stimulation pathway for PDGF.

1. Introduction

We have reported in Part 1 that human dermal fibroblasts in contarcted collagen gel did not multiply in response to EGF. The negative response to EGF is a distinct characteristic of the cells in contracted collagen gel, where the density of cell-collagen interaction sites may be as high as that in vivo. Thus the contracted collagen gel provides another quiescent state for fibroblasts. It is unknown whether human dermal fibroblasts growth-arrested in a contracted collagen gel multiply in response to other growth factors such as basic-fibroblast growth factor (b-FGF), platelet-derived growth factor (PDGF) or transforming growth factor-β (TGF-β).

These growth factors are defined as substances which are capable of reinitiating multiplication of cells in a quiescent state such as in a low serum condition or in cell-cell contact inhibition. Mitogenic effects of the growth factors have been found for various cells of epithelial or mesenchymal origin in culture (Antoniades and Owen, 1982; Carpenter and Cohen, 1984; Ross et al., 1986; Massaque, 1987; Kawamoto et al., 1989). Mitogenic response to the growth factors is initiated by the binding of a growth factor to the specific receptor, then the binding is believed to generate a series of signals in the membrane, cytosol and nucleus. The signal transduction comprises multiple steps including unidentified processes up to mitogenesis, and the pathways have been shown to depend on the kinds of growth factors (Sawyer and Cohen, 1981; Nishizuka, 1984; Rozengurt, 1986; Magnaldo et al., 1986; L'Allemain and Pouyssegur, 1986; Matuoka et al., 1988). It has been demonstrated that the proliferative response of cells to a growth factor is affected by the extracellular substrate (Gospodarowicz and Tauber, 1980; Rhudy and McPherson, 1988; Nakagawa et al., 1989). Therefore, it is intriguing whether the strong interactions between a cell and collagen fibrils, called cell-matrix contact inhibition, prevent the growth stimulation by other growth factors as well as by EGF, in that

the characteristics of cell-matrix contact inhibition could be defined in terms of the intracellular signal transduction induced by each growth factor.

In Part 2, the growth response to the growth factors, EGF, b-FGF, PDGF and TGF-β, was examined with human dermal fibroblasts in three different quiescent states; in a low serum condition, at confluency and in contracted collagen gel. Among the growth factors examined, only PDGF was found to stimulate the proliferation of fibroblasts growth-arrested in a contracted collagen gel.

2. Materials and Methods

Cell culture

Fibroblasts were isolated from skin of a 30-year-old female (HF-30) and a 41-year-old male (HF-41) obtained during plastic surgery, as described in Chapter I. Cultures at the 4th population doubling level (PDL) were frozen and kept in liquid nitrogen. Human fibroblasts from the 7th PDL to 10th PDL were used throughout the present experiments.

One ml of the cell suspension ($1x10^5$ cells/ml) in Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical Co., Tokyo) containing 10% fetal bovine serum (FBS, GIBCO Labs., New York) was incubated at 37 °C on a 24-well flat-bottomed plastic tray. The culture medium was renewed every 2 days.

For the culture with 0.2% FBS-DMEM, 1 ml of the cell suspension in 5% FBS-DMEM ($2x10^5$ cells/ml) was placed on a plastic dish and incubated at 37 °C for 6 h. The cultures were washed twice with 2 ml of 0.2% FBS-DMEM and then incubated with 0.2% FBS-DMEM for 24 h at 37 °C. After reaching the quiescent state, the cells were cultured with a growth factor at an indicated concentration for 9-72 h.

Preparation of contracted collagen gels containing fibroblasts

A solution of pepsin-treated type I collagen (porcine tendon) in dilute HCl (pH 3.0) at a concentration of 3 mg/ml was obtained from Nitta Gelatin Co. (Osaka).

Solutions of 6 ml of collagen solution (3 mg/ml), 3 ml of 3x concentrated DMEM, 1 ml of FBS, 5 ml of 10% FBS-DMEM and 3 ml of the cell suspension in 10% FBS-DMEM ($9x10^5$ cells/ml) were gently mixed at 4 °C giving the final density of $1.5x10^5$ cells/ml and the final collagen concentration of 1.0 mg/ml. One ml of the mixed solution containing cells and collagen was incubated in a bacteriological 24-well flat-bottomed plastic tray (Terumo Co., Tokyo) at 37 °C. Collagen gel formed was scraped off at the periphery at 3 h after starting the culture to release the gels from the wells so that the collagen gels contracted uniformly (Chapter I). The incubation was continued at 37 °C to day 8, when the cell multiplication ceased. The culture medium was renewed every 2 days. The fibroblasts growth-arrested in the contracted collagen gel were then cultured with a growth factor at an indicated concentration for 9-72 h.

Determination of cell number

The cell number on plastic dishes or in contracted collagen gels was determined from measurement of DNA content as described by Labarca and Paigen (1980), followed by its conversion to cell number by using the factor of 10 pg DNA/cell. Briefly, cells grown in contracted collagen gel were placed in a plastic tube and incubated with 0.2% bacterial collagenase (Wako Pure Chemical Co., Tokyo)-1 mM $CaCl_2$ in phosphate-buffered saline (PBS, pH 7.4) for 2 h to dissolve the gels. The floating cells were then collected by centrifugation at 300 x g for 10 min. The cell pellets were washed twice with 0.1% trypsin plus 0.02% EDTA in PBS. DNA contents were determined fluorometrically as described above (Labarca and Paigen, 1980).

Growth factors

EGF from mouse submaxillary glands was obtained from Sigma (St. Louis, MO). PDGF from human platelets, b-FGF from bovine brain and TGF-β from porcine platelets were purchased from R & D Systems Inc. (Minneapolis, MN).

3. Results

3.1 Growth response of quiescent fibroblasts on plastic dishes to growth factors

The growth responses to EGF, b-FGF, PDGF or TGF-β of human dermal fibroblasts (HF-30) in mitotic arrest on plastic dishes under a low serum condition (0.2% FBS) or in a confluent culture with 10% FBS are shown in Table IV-4 and Fig. IV-6. Quiescent fibroblasts cultured under a low serum condition began to multiply upon exposure to EGF, b-FGF or PDGF, but not to TGF-β. The non-dividing fibroblasts in confluent monolayer culture, under cell-cell contact inhibition, resumed multiplying upon exposure to each growth factor in the concentration range from 0.1 ng/ml to 100 ng/ml. Increases in the cell number after 60 h of incubation with EGF (30 ng/ml), b-FGF (10 ng/ml), PDGF (30 ng/ml) and TGF-β (30 ng/ml) were 35 ± 2 %, 59 ± 2 %, 51 ± 6 % and 33 ± 3 % of that in the control culture, respectively. The combination of any two or more growth factors more markedly stimulated the growth of fibroblasts than a single type of growth factor (Table IV-5).

Response to growth factors was essentially the same for human fibroblasts derived from the skin of another person (HF-41) growth-arrested under a low serum condition or in a confluent culture (data not shown).

3.2 Mitogenic effect of EGF, b-FGF, PDGF or TGF-β on human dermal fibroblasts growth-arrested in contracted collagen gel

Collagen gels containing human dermal fibroblasts in the presence of 10% serum contracted rapidly and by 8 days after the start of culture the fibroblasts in contracted collagen gels had ceased to divide (data not shown). The fibroblasts at 2 days after cessation of proliferation in the contracted gel were treated with EGF, b-FGF, PDGF or TGF-β to observe the mitogenic effect.

The time courses of the growth response to each growth factor (30 ng/ml) of the fibroblasts in contracted gels are shown in Fig. IV-7A. Quiescent fibroblasts responded to PDGF rapidly after incubation for 24 h, but not to the other growth factors during incubation for up to 72 h. Dose dependency of growth stimulation by the growth factors is shown in Fig. IV-7B, based on determination of the increase of cell number at 60 h after incubation with the growth factors in the concentration range from 0.1 ng/ml to 100 ng/ml. No growth stimulation by EGF, b-FGF or TGF-β on the fibroblasts growth-arrested in the contracted gels was seen at any concentration. Combinations of any two of these three growth factors were also ineffective (Table IV-5). Only PDGF was found to be potent in stimulating growth of the fibroblasts growth-arrested in the contracted gels at a concentration comparable to the concentration effective for the cells at confluency in monolayer culture (Fig. IV-6). Combinations of PDGF with one of the other growth factors also stimulated the growth of fibroblasts, but only to the same extent as in the case of PDGF alone, indicating that the other growth factors did not interact with the growth signal transduction by PDGF (Table IV-5).

The growth response to PDGF was also observed for the human fibroblasts derived from the skin of another person (HF-41) growth-arrested in contracted collagen gels (data not shown).

4. Discussion

In the present study, we found that in contrast to the fibroblasts in quiescent states owing to either serum depletion or cell-cell contact inhibition, the quiescent cells in the contracted collagen gels were stimulated by PDGF but not by EGF, b-FGF and/or TGF-β. We propose that the negative mitogenic response to EGF, b-FGF or TGF-β of the fibroblasts in contracted collagen gels is due to the high density and ubiquitous distribution of collagen fibril-cell membrane interaction sites. We hypothesized that the repression of cell growth in the contracted gel was not due to direct cell-cell contact, but was due to the distribution and number of contact sites between the cells and surrounding collagen fibrils (Chapter III and Part A ; Nishiyama et al., 1989; Tsunenaga et al., 1989) or cell-matrix contact inhibition. We found that fibroblasts surrounded by a three-dimensional structure of collagen fibrils, particularly in the contracted gel, were repressed in the growth response to EGF, while the cells grown on a two-dimensional surface of collagen gel were not (Part A). It was also shown that EGF bound to the receptors of fibroblasts in contracted collagen gels as strongly as to those of the cells on a plastic dish, in that the number of binding sites and the apparent binding constant were unchanged. Therefore, the strong suppression of the mitotic response to EGF, b-FGF or TGF-β of fibroblasts in contracted gels might be related to interference with the signal transductions of these growth factors by collagen fibrils. Specifically, transduction of the signal delivered by the binding requires rapid phosphorylation, redistributions in the plane of the membrane and endocytosis (Rozengurt, 1986). Modifications of these processes induced by the interaction between the cell membrane (via collagen receptors) and collagen fibrils could result in the strong suppression of the growth response to EGF, b-FGF or TGF-β.

Characteristics of growth factor actions are summarized

in Table IV-6. Several features are similar to each other, but a signal transduction pathway for PDGF is distinct from that of other growth factors. In this respect, the finding in the present study that only PDGF could activate the proliferation of fibroblasts growth-arrested in contracted collagen gels may suggest a distinct pathway of siganl transduction of PDGF as compared with other growth factors. The phenomenon demonstrated that the quiescent state of fibroblasts in the contracted gels was different from that under a low serum condition or in confluent monolayer culture. The previous reports concerning signaling pathways induced by growth factors using fibroblast cultures on plastic indicated that PDGF acts through a pathway that was different from those activated by EGF, b-FGF or serum (Sawyer and Cohen, 1981; Nishizuka, 1984; Rozengurt, 1986; Magnaldo et al., 1986; L'Allemain and Pouyssegur, 1986; Matuoka et al., 1988). Treatment of fibroblasts with PDGF rapidly elicited enzymatic hydrolysis of phosphatidyl-inositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol, both of which are intracellular second messengers. It has recently been reported that PIP_2 breakdown appears to be a crucial process in the initiation and maintenance of cell proliferation induced by PDGF (Matuoka et al., 1988). In contrast, mitogenesis of fibroblasts by EGF, b-FGF or serum is independent of PIP_2 breakdown (Magnaldo et al., 1986; L'Allemain and Pouyssegur, 1986; Matuoka et al., 1988). An alternative pathway of signal transduction for PDGF may well exist. The present findings together with the previous reports may suggest that in the contracted gels the high level of interaction of collagen fibrils with the cell membrane did not abolish the polyphosphoinositide signaling pathway induced by PDGF, whereas it completely repressed the other signaling pathway activated by EGF, b-FGF or serum.

Further evidence for distinctness of the quiescent state of fibroblasts in contracted collagen gel was obtained in the response of cells to growth factors as shown in this chapter.

The effect of PDGF was different from those of other growth factors including EGF, b-FGF and TGF-β on the stimulation of proliferation. Furthermore, it was found that a combination of EGF, b-FGF or TGF-β with a particular saponin, Saikosaponin b_1, initiated the cellular multiplication in contracted collagen gel, though as described above EGF, b-FGF or TGF-β alone did not show the mitogenic effect. These results suggest that the quiescent state of the fibroblasts in contracted collagen gel at a high density of interactions between a cell membrane (via cell surface receptors) and collagen fibrils is mainly caused by the suppression of certain signal transduction pathway(s) of growth stimulation, which might inhibit the weakening of cell-collagen adhesion (or binding) induced by growth factors. PDGF and saikosaponin b_1 might be useful for studying the mechanisms of cell-matrix contact inhibition by the strong interactions of cell surface receptors with collagen fibrils.

Finally, the quiescent fibroblasts in the contracted collagen gel may be useful as a research tool, comprising a model system with a limited, specific signaling pathway, for investigating cell growth regulation.

Table IV-4. Growth response to growth factor of quiescent fibroblasts on plastic under a low serum condition

Growth Factor	Growth Stimulation (% of Control)
EGF (24 ng/ml)	145 ± 10
b-FGF (10 ng/ml)	148 ± 3
PDGF (30 ng/ml)	143 ± 4
TGF-β (10 ng/ml)	90 ± 4

Human dermal fibroblasts (HF-30) were cultured in 0.2% FBS-DMEM containing a growth factor for 72 h. The data are expressed as % cell number with respect to the control culture without growth factor and each value is the mean of triplicate determinations ± SD.

Table IV-5. Growth response of human fibroblasts to combinations of growth factors

Growth Factor	Growth Stimulation (% of Control) on Plastic	in Contracted Gel
EGF + b-FGF	203 ± 1	105 ± 1
EGF + PDGF	187 ± 2	128 ± 3
EGF + TGF-β	159 ± 3	107 ± 3
b-FGF + TGF-β	184 ± 4	105 ± 3
EGF + b-FGF + PDGF + TGF-β	223 ± 2	128 ± 2

Human dermal fibroblasts (HF-30) growth-arrested in confluent culture on plastic dishes or in contracted collagen gels were treated with combinations of growth factors for 60 h. The data are expressed as % cell number with respect to the control culture without growth factors and each value is the mean of triplicate determinations ± SD. Concentration of growth factors : EGF 24 ng/ml; b-FGF 10 ng/ml; PDGF 30 ng/ml; TGF-β 10 ng/ml.

Table IV-6. Some growth factors and their actions

	EGF	b-FGF	PDGF	TGF-β
Stimulation of cell growth	+	+	+	+
Competence factor (G_0 --> G_1)	+	+	+	+
Binding to its receptor	+	+	+	+
Internalization of GF-R complex	+	+	+	+
Tyrosin kinase activity	+	+	+	+
Phosphoinositide (PI) turnover	-	-	+	-
Weakening of cell adhesion	+	+	+	+
Cell motility (chemotaxis)	+	+	+	+

(+)= property or activity present.
(-)= property or activity absent.

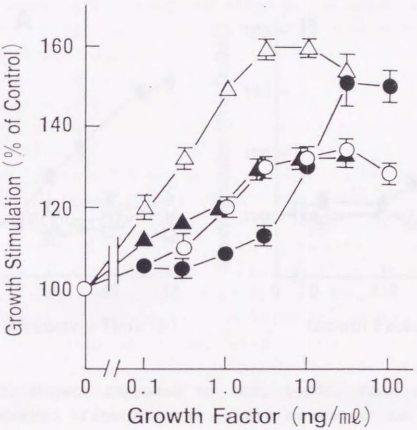

Fig.IV-6. Growth response to EGF, b-FGF, PDGF or TGF-β of human dermal fibroblasts at confluency on plastic dishes. Fibroblasts (HF-30) were grown on plastic dishes in 10% FBS-DMEM. Cultures were used 2 days after the cessation of proliferation. At this stage, each growth factor at the concentration indicated was added to 10% FBS-DMEM. After incubation for 60 h, the cell number was determined as described in Materials and Methods. The data are expressed as % cell number with respect to the control culture in the medium without any growth factor. Each point represents the mean of triplicate determinations ± SD. ○ EGF; △ b-FGF; ● PDGF; ▲ TGF-β.

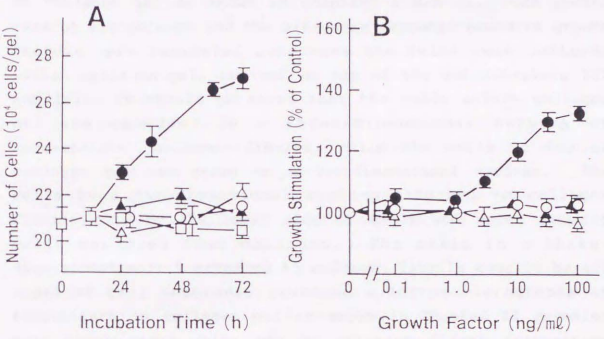

Fig.IV-7. Growth response to EGF, b-FGF, PDGF or TGF-β of human dermal fibroblasts growth-arrested in contracted collagen gel. Fibroblasts (HF-30) in contracted gel were cultured in 10% FBS-DMEM. Cultures were used 2 days after the cessation of proliferation. At this stage, each growth factor was added to 10% FBS-DMEM. A: time course curves of growth response to 30 ng/ml of growth factor. Control(□) ; EGF(○) ; b-FGF(△); PDGF(●); TGF-β(▲). The cell number was determined as described in Materials and Methods. Each point represents the mean of triplicate determinations ± SD. B: dose-dependency of growth response to each growth factor after incubation for 60 h. The data are expressed as % cell number with respect to the control culture in the medium without any growth factor. Each point represents the mean of triplicate determination ± SD. EGF(○); b-FGF(△); PDGF(●); TGF-β(▲).

General Discussion

The morphology and the cytoskeletal organization of human dermal fibroblasts within collagen gel were distinguished from those on plastic (or on glass), on collagen-coated dishes and on collagen gel as shown in Chapters I and II. The growth rate of fibroblasts and the mitogenic responsiveness to growth factors were repressed only when the cells were cultured within collagen gel, but not on top of the gel (Chapters III and IV). It should be noted that the cells within collagen gel are embedded in a three-dimensional network of reconstituted collagen fibrils, while the cells on top of collagen gel are grown on a two-dimensional surface. The cells on a two-dimensional surface attached to collagen fibrils only by the lower side of cells and upper side of cells was free from collagen. The cells in a three-dimensional matrix attached to collagen fibrils equally by all sides of cell membrane. Scanning electron micrographs of fibroblasts in collagen gel as shown in Chapter II revealed many rough picks which can be putative direct interaction sites between a cell and collagen fibrils. The resultant difference in cellular shape, cytoskeletal organization, cell growth rate and growth response to growth factors could have resulted from the distribution of cell-collagen fibril interaction sites per cell.

The elongation process of fibroblasts in collagen gel is induced by the interactions. The morphological change of fibroblasts in collagen gel from initial spherical shape was markedly retarded as compared to that on glass and on collagen gel. The retardation in collagen gel was found to be antagonized upon treatment with a low dose of cytochalasin D (0.2 μM), accompanying disorganization of actin microfilaments to fragments. In addition, the extremely elongate cell shape caused by collagen gel was not changed upon treatment with cytochalasin D. From the results in Chapter II, the role of actin microfilaments under the influence of interaction with

collagen fibrils across the plasma membrane are elucidated; 1) actin microfilaments hold the scaffold of initial spherical cells and prevent the following elongation process, and 2) actin microfilaments do not participate in maintenance of elongated fibroblasts. Therefore, the elongate shape of fibroblasts might be maintained by the interactions in a linkage of collagen--collagen receptors--cytoskeleton other than the well known linkage of fibronectin--integrin--(talin, vinculin, α-actinin)--actin microfilaments (Horwitz et al., 1986; Burridge et al., 1988).

The cellular behavior within collagen gel which was examined in the present study might be closely coupled with each other and influenced by the interactions between a cell and collagen fibrils. The interactions observed in the present study are thought to be mediated through specific collagen receptors directly. Collagen is known to bind with other extracellular matrix components such as fibronectin. Involvement of fibronectin in the present observations cannot be ruled out. The issue resides out of the present examination.

Our observation of the distinct cellular behavior within three-dimensional collagen gel leads to a hypothesis that the distribution of cell-collagen interaction sites per cell is one of the intrinsic factors for regulation of cellular activity. That is, the effect of extracellular matrix on cellular behavior is influenced by the state of interactions with cells, even if components of extracellular matrix are biochemically identical.

The human dermal fibroblasts in contracted collagen gel containing high density of collagen fibrils stopped multiplying, even in the presence of 10% fetal bovine serum. The inhibition of cell growth caused by collagen fibrils in three-dimensional collagen matrix at a high density of cell-collagen fibril interaction sites per cell was found, not due to direct cell-cell contact inhibition, not due to repression of general cellular activity, not due to reduction of

diffusion of nutrients including growth factors, nor due to masking of growth factor-receptor as shown in Chapters III and IV. Characteristics of this quiescent state of the fibroblasts cultured in the contracted collagen gel were clearly demonstrated in terms of the mitogenic responsiveness to growth factors. Quiescent fibroblasts in contracted collagen gel were stimulated in growth only by PDGF, but not by EGF, b-FGF and TGF-β. This result demonstrates that collagen fibrils at a high density of cell-collagen fibril interaction sites per cell completely prevent the intracellular signal transduction pathway of growth factor stimulation except for PDGF.

Our study of the inhibition of normal fibroblast proliferation in contracted collagen gel (Chapters III and IV) leads to another hypothesis. It is that the high density of collagen fibrils in contracted collagen gel, resulting in a high level of interaction between a cell and collagen fibrils, is the major reason for the growth inhibition and the strong suppression of mitogenic responsiveness to growth factors. In this respect, preliminary studies on human fibrosarcoma HT1080 cells are worth referring to reinforce the hypothesis. Many tumor cells not merely divide without anchorage but also fail to make firm attachments to the extracellular matrix. It was reported that when fibroblasts from a chick embryo are transformed with v-src, they secrete large amounts of plasminogen activator, which loosens their attachments to the culture dishes. When these cells were grown in the presence of an antibody that blocks the activity of this protease, they attach more firmly to the dish and, at the same time, become more obedient to the normal social controls on cell proliferation: instead of piling up in multiple layers, they tend to stop dividing on reaching confluence (Sullivan and Quigley, 1986; Alberts et al., eds., 1989-a).

Thus, for these transformed cells, the formation of firm attachments to the extracellular matrix seems to inhibit growth. In the case of HT1080 cells, it has been reported

that the cells have the collagen-specific receptors (Wayner and Carter, 1987). We have observed that HT1080 cells within collagen gel showed attachment to collagen fibrils and cellular shape similar to human dermal fibroblasts, but collagen gel contraction by HT1080 cells was weak and the growth rate of HT1080 cells was not repressed within collagen gel or slightly contracting gel (Nishiyama et al., in contribution). On the contrary, the growth of HT1080 cells was inhibited in contracted collagen gel when cells were co-cultured with normal fibroblasts. In the co-culture, collagen gel contraction was as marked as that by normal fibroblasts alone. In the co-culture of HT1080 cells and normal fibroblasts, firm attachments by large number of interaction sites between cell and collagen fibrils might have been formed, resulting in inhibiting proliferation of HT1080 cells. The high density of collagen fibrils in contracted collagen gel which is mediated through the fibroblasts, resulting in large number of interaction sites between a fibroblast and collagen fibrils, is a major reason for the new quiescent state of fibroblasts in contracted collagen gel.

Three-dimensional organization of collagen fibrils provides a potent solid-phase regulator for fibroblast activities. The quiescent fibroblasts in the contracted collagen gel may be useful as a research tool, comprising a model system with a limited, specific signaling pathway, for investigating cell growth regulation.

Conclusion and Perspective

In the present thesis, a distinct characteristic of the cellular behavior of human dermal fibroblasts within a three-dimensional matrix of reconstituted collagen fibrils was demonstrated in comparison with the cells on a two-dimensional surface of the matrix. The difference was interpreted to be due to the ubiquitous distribution and/or high density of collagen-cell interaction sites on the entire surface of the cell. The morphology, cytoskeletal organization, cell growth rate and mitogenic responsiveness to growth factors were closely correlated with each other. The influence from the interactions between cell and collagen fibrils may have some common signal transduction of the influence from collagen fibrils. These initial sites must be collagen receptors in the cell membrane. The present observations are essentially identical as to regulatory effects of collagen matrix on cellular activity, including the reduced protein and collagen synthesis by the fibroblasts in the contracted collagen gel as previously reported. From the evidence on direct action of collagen fibrils to human dermal fibroblasts, three-dimensional organization of collagen fibrils provides a potent solid-phase regulator for cellular activities.

In any case, flexibility of the collagen aggregates is one of the important characteristics of this protein and the collagen is a potent biomaterial not only just as building blocks for construction of scaffold architecture of living organisms but also as solid-phase regulators for multicellular activities. It should be further pointed out that the metabolic charcteristics of collagen, stable yet degradable, might be important in designing multicellular architecture analogous to living tissues and organs.

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References

Abe, H., Sakaguchi, M., Konishi, H., Tani, T. and Arichi, S.: The effects of saikosaponins on biological membranes. 1. The relationship between the structures of saikosaponins and haemolytic activity. Planta Medica 34: 160-166, 1978.

Abe, H., Sakaguchi, M., Yamada, M., Arichi, S. and Odashima, S.: Pharmacological actions of saikosaponins isolated from Bupleurum falcatum. 1. Effects of saikosaponins on liver function. Planta Medica 40: 366-372, 1980.

Adams, L.W. and Priestley, G.C.: Contraction of collagen lattices by skin fibroblasts from dystrophic recessive epidermolysis bullosa and other dermatoses. J. Invest. Dermatol. 87: 544-546, 1986.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., Watson, J.D.: Molecular biology of the cell, 2nd ed., pp.727-790 (a), 791-836 (b), 951-1001 (c), 1122-1136 (d), Garland Publishing, Inc., New York, 1989.

Akiyama, S.K., Nagata, K. and Yamada, K.M.: Cell surface receptors for extracellular matrix components. Biochim. Biophys. Acta 1031: 91-110, 1990.

Antoniades, H.N. and Owen, A.J.: Growth factors and regulation of cell growth. Ann. Rev. Med. 33: 445-463, 1982.

Barrandon, Y. and Green, H.: Three clonal types of keratinocyte with different capacities for multiplication. Proc. Natl. Acad. Sci. USA 84: 2302-2306, 1987.

Bartley, W., Birt, L.M. and Banks, P.: "The biochemistry of the tissues", John Wiley and Sons, London, p.225-234, 1968.

Bell, E., Ivarsson, B. and Merrill, C.: Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro. Proc. Natl. Acad. Sci. USA 76: 1274-1278, 1979.

Burden, S.J., Sargent, P.B. and McMahan, U.J.: Acetylcholine receptors in regenerating muscle accumulate at original synaptic sites in the absence of the nerve. J. Cell Biol. 82: 412-425, 1979.

Burridge, K., Fath, K., Kelly, T., Nuckolls, G. and Turner, C.: Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. Annu. Rev. Cell Biol. 4: 487-526, 1988.

Buttle, D.J. and Ehrlich, H.P.: Comparative studies of collagen lattice contraction utilizing a normal and transformed cell line. J. Cell. Phys. 116: 159-166, 1983.

Carpenter, G. and Cohen, S.: ^{125}I-labeled human epidermal growth factor: binding, internalization and degradation in human fibroblasts. J. Cell Biol. 71: 159-171, 1976.

Carpenter, G. and Cohen, S.: Peptide growth factors. Trends Biochem. Sci. 9: 169-171, 1984.

Carter, W.G. and Wayner, E.A.: Characterization of the class III collagen receptor, a phosphorylated, transmembrane glycoprotein expressed in nucleated human cells. J. Biol. Chem. 263: 4193-4201, 1988.

Colige, A., Nusgens, B. and Lapiere, C.M.: Effect of EGF on human skin fibroblasts is modulated by the extracellular matrix. Arch. Dermatol. Res. 280: s42-s46, 1988.

Coulomb, B., Dubertret, L., Merrill, C., Fosse, M., Breton-Gorius, J., Prost, C. and Touraine, R.: Endogenous peroxidases in normal human dermis : a marker of fibroblast differentiation. J. Invest. Dermatol. 81: 75-78, 1983.

Cotta-Pereira, G., Rodrigo, F.G. and David-Ferreira, J.F.: The use of tannic acid-glutaraldehyde in the study of elastic and elastic-related fibers. Stain Technol. 51: 7-11, 1976.

Delvoye, P., Nusgens, B. and Lapière, C.M.: The capacity of retracting a collagen matrix is lost by dermatosparactic skin fibroblasts. J. Invest. Dermatol. 81: 267-270, 1983.

Delvoye, P., Mauch, C., Krieg, T. and Lapière, C.M.: Contraction of collagen lattices by fibroblasts from patients and animals with heritable disorders of connective tissue. Br. J. Dermatol. 115: 139-146, 1986.

Ehrlich, H.P., Buttle, D.J., Trelstad, R.L. and Hayashi, K.: Epidermolysis bullosa dystrophica recessive fibroblasts altered behavior within a collagen matrix. J. Invest.

Dermatol. 80: 56-60, 1983.

Ehrlich, H.P. and Wyler, D.W.: Fibroblast contraction of collagen lattices in vitro : Inhibition by chronic inflammatory cell mediators. J. Cell. Phys. 116: 345-351, 1983.

Ehrlich, H.P., Borland, K.M., Muffly, K.M. and Hall, P.F.: Contraction of collagen lattice by peritubular cells from rat testis. J. Cell Sci. 82: 281-294, 1986(a).

Ehrlich, H.P., Griswold, T.R. and Rajaratnam, J.B.M.: Studies on vascular smooth muscle cells and dermal fibroblasts in collagen matrices. Effects of heparin. Exp. Cell Res. 164: 154-162, 1986(b).

Elsdale, T. and Bard, J.: Collagen substrata for studies on cell behavior. J. Cell Biol. 54: 626-637, 1972.

Gabbiani, G. and Rungger-Brandle, E.: The fibroblast. In Tissue Repair and Regeneration (L.E. Glynn, ed.), pp. 1-50. Handbook of inflammation, Vol. 3. Amsterdam: Elsevier, 1981.

Gillery, P., Maquart, F.X. and Borel, J.P.: Fibronectin dependence of the contraction of collagen lattices by human skin fibroblasts. Exp. Cell Res. 167: 29-37, 1986.

Gospodarowicz, D. and Tauber, J.: Growth factors and the extracellular matrix. Endocrine Rev. 1: 201-227, 1980.

Green, H.: Terminal differentiation of cultured human epidermal cells. Cell 11: 405-415, 1977.

Grinnell, F. and Lamke, C.R.: Reorganization of hydrated collagen lattices by human skin fibroblasts. J. Cell Sci. 66: 51-63, 1984.

Guidry, C. and Grinnell, F.: Studies on the mechanism of hydrated collagen gel reorganization by human skin fibroblasts. J. Cell Sci. 79: 67-81, 1985.

Guidry, C. and Grinnell, F.: Contraction of hydrated collagen gels by fibroblasts : evidence for two mechanisms by which collagen fibrils are stabilized. Collagen Rel. Res. 6: 515-529, 1986.

Guidry, C. and Grinnell, F.: Heparin modulates the organization of hydrated collagen gels and inhibits gel

contraction by fibroblasts. J. Cell Biol. 104: 1097-1103, 1987.

Gullberg, D., Tingstrom, A., Thuresson, A.C., Olsson, L., Terracio, L., Borg, T.K. and Rubin, K.: β_1 Integrin-mediated collagen gel contraction is stimulated by PDGF. Exp. Cell Res. 186: 264-272, 1990.

Harkness, M.L.R., Harkness, R.D. and James, D.W.: The effect of a protein-free diet on the collagen content of mice. J. Physiol. 144: 307-313, 1958.

Hata, R., Sunada, H., Arai, K., Sato, T., Ninomiya, Y., Nagai, Y. and Senoo, H.: Regulation of collagen metabolism and cell growth by epidermal growth factor and ascorbate in cultured human skin fibroblasts. Eur. J. Biochem. 173: 261-267, 1988.

Hata, R. and Seno, H.: L-Ascorbic acid 2-phosphate stimulates cellagen accumulation, cell proliferation, and formation of a three-dimensional tissuelike substance by skin fibroblasts. J. Cell. Physiol. 138: 8-16, 1989.

Herman, B. and Pledger, W.J.: Platelet-derived growth factor-induced alternations in vinculin and actin distribution in BALB/c-3T3 cells. J. Cell Biol. 100: 1031-1040, 1985.

Herman, B., Harrington, M.A., Olashaw, N.E. and Pledger, W.J.: Identification of the cellular mechanisms responsible for platelet-derived growth factor induced alterations in cytoplasmic vinculin distribution. J. Cell. Physiol. 126: 115-125, 1986.

Horwitz, A., Duggan, K., Buck, C., Beckerle, M.C. and Burridge, K.: Interaction of plasma membrane fibronectin receptor with talin-a transmembrane linkage. Nature 320: 531-533, 1986.

Hynes, R.O.: Integrins: a family of cell surface receptor. Cell 48: 549-554, 1987.

Kawamoto, T., Nishi, M., Takahashi, K., Nishiyama, T., Sato, J.D. and Taniguchi, S.: Stimulation by transforming growth factor-β of epidermal growth factor-dependent growth of aged human fibroblasts: recovery of high affinity EGF receptors

and growth stimulation by EGF. In Vitro Cell. Dev. Biol. 25: 965-970, 1989.

Kleinman, H.K., Klebe, R.J. and Martin, G.R.: Role of collagenous matrices in the adhesion and growth of cells. J. Cell Biol. 88: 473-485, 1981.

Labarca, C. and Paigen, K.: A simple, rapid and sensitive DNA assay procedure. Anal. Biochem. 102: 344-352, 1980.

L'Allemain, G. and Pouyssegur, J.: EGF and insulin action in fibroblasts : evidence that phosphoinositide hydrolysis is not an essential mitogenic signalling pathway. FEBS Lett. 197: 344-348, 1986.

Magnaldo, I., L'Allemain, G., Chambard, J.C., Moenner, M., Barritault, D, and Pouyssegur, J.: The mitogenic signaling pathway of fibroblast growth factor is not mediated through polyphosphoinositide hydrolysis and protein kinase C activation in hamster fibroblasts. J. Biol. Chem. 261: 16916-16922, 1986.

Massague, J.: The TGF-β family of growth and differentiation factors. Cell 49: 437-438, 1987.

Matuoka, K., Fukami, K., Nakanishi, O., Kawai, S., and Takenawa, T.: Mitogenesis in response to PDGF and bombesin abolished by microinjection of antibody to PIP_2. Science 239: 640-643,1988.

Mauch, C., Hatamochi, A., Scharffetter, K. and Krieg, T.: Regulation of collagen synthesis in fibroblasts within a three-dimensional collagen gel. Exp. Cell Res. 178: 493-503, 1988.

Nakagawa, S., Pawelek, P. and Grinnell, F.: Extracellular matrix organization modulates fibroblast growth and growth factor responsiveness. Exp. Cell Res. 182: 572-582, 1989.

Nishiyama, T., Tominaga, N. and Hayashi, T.: Collagen gel retraction by fibroblasts. Commun. in Applied Cell Biol. (Japan). 4: 1-6, 1985.

Nishiyama, T., Tominaga, N., Nakajima, K. and Hayashi, T.: Quantitative evaluation of the factors affecting the process of fibroblast-mediated collagen gel contraction by

separating the process into three phases. Collagen Rel. Res. 8, 259-273, 1988.

Nishiyama, T., Tsunenaga, M., Nakayama, Y., Adachi, E. and Hayashi, T.: Growth rate of human fibroblasts is repressed by the culture within reconstituted collagen matrix but not by the culture on the matrix. Matrix, 9: 193-199, 1989.

Nishiyama, T., Horii, I., Nakayama, Y., Ozawa, T. and Hayashi, T.: A distinct characteristic of the quiescent state of human dermal fibroblasts in contracted collagen gel as revealed by no response to epidermal growth factor alone, but a positive growth response to a combination of the growth factor and saikosaponin b_1. Matrix, 10: 412-419, 1990.

Nishiyama, T., Akutsu, N., Horii, I., Nakayama, Y., Ozawa, T. and Hayashi, T.: Response to growth factors of human dermal fibroblasts in a quiescent state owing to cell-matrix contact inhibition. Matrix, 11: in press, 1991.

Nishiyama, T., Akutsu, N., Horii, I. and Hayashi, T.: Distinctive cellular behaviors of human fibrosarcoma cells within collagen gel from human dermal fibroblasts. submitted for publication in Tissue Culture Res. Commun. (Tokyo).

Nishizuka, Y.: The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature (London) 308: 693-698, 1984.

Nusgens, B., Merrill, C., Lapière, C. and Bell, E.: Collagen biosynthesis by cells in a tissue equivalent matrix in vitro. Collagen Rel. Res. 4: 351-364, 1984.

Paye, M., Nusgens, B.V. and Lapiere, C.M.: Modulation of cellular biosynthetic activity in the retracting collagen lattice. Eur. J. Cell Biol. 45: 44-50, 1987.

Rhudy, R.W. and McPherson, J.M.: Influence of the extracellular matrix on the proliferative response of human skin fibroblasts to serum and purified platelet-derived growth factor. J. Cell. Physiol. 137: 185-191, 1988.

Ross, R., Raines, E.W. and Bowen-Pope, D.F.: The biology of

platelet-derived growth factor. Cell 46: 155-169, 1986.

Rozengurt, E.: Early signals in the mitogenic response. Science 234: 161-166, 1986.

Saiag, P., Coulomb, B., Lebreton, C., Bell, E. and Dubertret, L.: Psoriatic fibroblasts induce hyerproliferation of normal keratinocytes in a skin equivalent model in vitro. Science 230: 669-672, 1985.

Sarber, R., Hull, B., Merrill, C., Soranno, T. and Bell, E.: Regulation of proliferation of fibroblasts of low and high population doubling levels grown in collagen lattices. Mech. Ageing Dev. 17: 107-117, 1981.

Sato, J.D., Kawamoto, T., Le, A.D., Mendelsohn, J., Polikoff, J. and Sato, G.H.: Biological effects in vitro of monoclonal antibodies to human epidermal growth factor receptors. Mol. Biol. Med. 1: 511-529, 1983.

Sawyer, S.T. and Cohen, S.: Enhancement of calcium uptake and phosphatidylinositol turnover by epidermal growth factor in A-431 cells. Biochemistry 20: 6280-6286, 1981.

Schlessinger, J. and Geiger, B.: Epidermal growth factor induces redistribution of actin and α-actinin in human epidermal carcinoma cells. Exp. Cell Res. 134: 273-279, 1981.

Schor, S.L.: Cell proliferation and migration on collagen substrata in vitro. J. Cell Sci. 41: 159-175, 1980.

Schor, S.L., Schor, M., Winn, B. and Rushton, G.: The use of three-dimensional collagen gels for study of tumour cell invasion in vitro: experimental parameters influencing cell migration into the gel matrix. Int. J. Cancer 29: 57-62, 1982.

Shuster, S., Black, M.M. and McVitie, E.: The influence of age and sex on skin thickness, skin collagen and density. Br. J. Dermatol. 93: 639-643, 1975.

Steinberg, B.M., Smith, K., Colozzo, M. and Pollack, R.: Establishment and transformation diminish the ability of fibroblasts to contract a native collagen gel. J. Cell Biol. 87: 304-308, 1980.

Sugrue, S.P. and Hay, E.D.: Response of basal epithlial cell surface and cytoskeleton to solubilized extracellular matrix molecules. J. Cell Biol. 91: 45-54, 1981.

Sullivan, L.M. and Quigley, J.P.: An anticatalytic monoclonal antibody to avian plasminogen activator: its effect on behavior of RSV-transformed chick fibroblasts. Cell 45: 905-915, 1986.

Tanaka, K. and Naguro, T.: High resolution scanning electron microscopy of cell organelles by a new specimen preparation method. Biomed. Res. 2: 63-70, 1981.

Tanaka, K. and Mitsushima, A.: A preparation method for observing intracellular structures by scanning electron microscopy. J. Microsc. 133: 213-222, 1984.

Timpl, R.: Structure and biological activity of basement membrane proteins. Eur. J. Biochem. 180: 487-502, 1989.

Tomasek, J.J., Hay, E.D. and Fujiwara, K.: Collagen modulates cell shape and cytoskeleton of embryonic corneal and fibroma fibroblasts. Distribution of actictin, α-actinin and myosin. Dev. Biol. 92: 107-122, 1982.

Tomasek, J.J. and Hay, E.D.: Analysis of the role of microfilaments and microtubules in acquisition of bipolarity and elongation of fibroblasts in hydrated collagen gels. J. Cell Biol. 99: 536-549, 1984.

Tsunenaga, M., Akutsu, N., Nishiyama, T., Nakayama, Y., Adachi. E., and Hayashi, T.: Effect of three-dimensional collagen matrix on morphology of fibroblasts: role of actin filaments. Cell Struct. Funct. 14: 902, 1989.

Yoshizato, K., Taira, T. and Shioya, N.: Collagen-dependent growth suppression and changes in the shape of human dermal fibroblasts. Ann. Plast. Surg. 13: 9-14, 1984.

Yoshizato, K., Taira, T. and Yamamoto, N.: Growth inhibition of human fibroblasts by reconstituted collagen fibrils. Biomed. Res. 6: 61-71, 1985.

Yurchenco, P.D. and Ruben, G.C.: Basement membrane structure in situ: evidence for lateral associations in the type IV collagen network. J. Cell Biol. 105: 2559-2568, 1987.

Van Bockxmeer, F.M., Martin, C.M. and Constrable, I.J.: Effect of cyclic AMP on cellular contractility and DNA synthesis in chorioretinal fibroblasts maintained in collagen matrices. Exp. Cell Res. 155: 413-421, 1984.

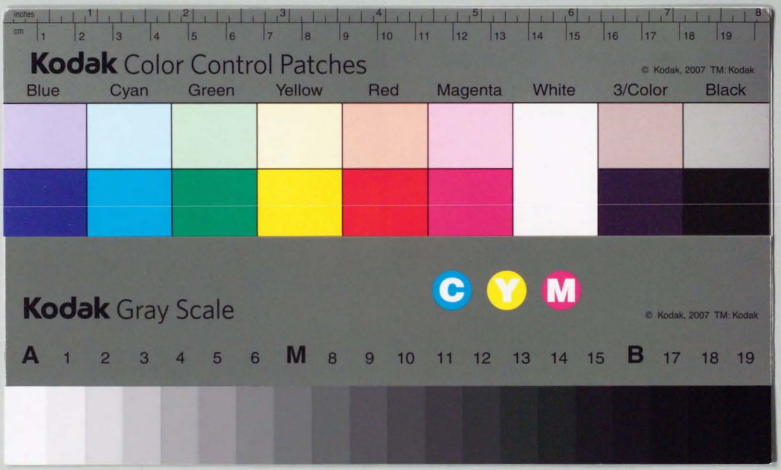
Van Gansen, P., Pays, A. and Malherbe, L.: Actin content and organization of microfilaments in primary cultures of mouse embryonic fibroblasts (in vitro ageing). Biol. Cell 54: 251-260, 1985.

Wang, E. and Gundersen, D.: Increased organization of cytoskeleton accompanying the aging of human fibroblasts in vitro. Exp. Cell Res. 154: 191-202, 1984.

Watt, F.M.: Selective migration of terminally differentiating cells from the basal layer of cultured human epidermis. J. Cell Biol. 98: 16-21, 1984.

Watt, F.M.: The extracellular matrix and cell shape. Trends Biochem. Sci. 11: 482-485, 1986.

Wayner, E.A. and Carter, W.G. : Identification of multiple cell adhesion receptors for collagen and fibronectin in human fibrosarcoma cells prossessing unique α and common β subunits. J. Cell Biol. 105: 1873-1884, 1987.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