GROWTH INHIBITION OF HUMAN FIBROBLASTS BY RECONSTITUTED COLLAGEN FIBRILS

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ABSTRACT

Experiments with fibroblasts in culture demonstrated that collagen fibrils strongly inhibited DNA synthesis, and suppressed cell growth. Collagen fibrils also induced a specific cell shape with abundant cytoplasmic protrusions. The rate of DNA synthesis on collagen was less than 20% of that on tissue culture plastic. The extent of the suppression of DNA synthesis was strictly dependent upon the quantity of collagen available to cells. Minimum effective quantity of collagen for the suppression of DNA synthesis was 60 pg/cell, which differed from that for the induction of a collagen-mediated change in the cell shape (5.3 ng/cell). The extent of suppression of DNA synthesis on collagen fibrils decreased as the length of culture period increased, suggesting that fibroblasts interact with collagen during culture and induce changes in the properties of collagen. Native collagen which loses ability to form a fibril in the culture condition was prepared by modifying ϵ -amino groups of lysyl residues. The chemically modified collagen did not induce any collagen-mediated changes in fibroblasts, indicating the importance of a polymeric form of collagen for collagen to cell interaction.

Studies on cell-to-collagen interactions have a relatively long history. Early works were done chiefly by Huzella and associates, who studied the behavior of cells on fibrous collagen (5, 10). Ehrmann and Gey made a systematic study on the use of collagen gels for supporting cell growth (1). They found that collagen gel proved superior to the bare glass for the growth of various cell strains and fresh tissue explants. Since these studies, it has been generally accepted that collagen used as a substratum in cell culture is useful for improving cell growth and cell development (4).

Elsdale and Bard devised a simple technique

for the preparation of collagen substrata (hydrated collagen lattices: HCLs) and studied cell morphology and cell growth on HCLs (2). They noticed suppression of DNA synthetic activity of fibroblasts grown on HCLs as compared to fibroblasts on plastic, which seems to apparently contradict a generally accepted role of collagen in a cell culture system.

We found that DNA synthesis in normal human skin fibroblasts was remarkably reduced on collagen-coated dishes as compared with that on tissue culture plastic (15). The effect of collagen manifests only when collagen holds its native structure.

We have extended further the study on the collagen-mediated changes in the cell. In the present paper, we describe detailed studies of the effect of collagen on DNA synthesis in human fibroblasts, changing number of cells, quantity of collagen, length of culture period, and the chemi-

Abbreviations: EDTA, ethylenediaminetetraacetate; HEPES, *N*-2-hydroxypiperazine-*N'*-2-ethansulfonic acid; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

cal nature of collagen. It is clearly shown that a reconstituted collagen fiber is a potent suppressor of DNA synthesis in human dermal fibroblasts. The probable physiological meaning of the collagen-mediated suppression of the cell growth is discussed considering the *in vivo* status in which dermal fibroblasts reside.

MATERIALS AND METHODS

Chemicals and culture materials were obtained as follows. EDTA and HEPES from Dojin Chemical Institute (Kumamoto). Fetal calf serum (FCS) from Commonwealth Serum Laboratory (Melbourne, Australia). Trypsin, calf thymus DNA and pepsin from Sigma Chemical (St. Louis, MO, U.S.A.). Crude bacterial collagenase from Wako Pure Chemical Industries (Tokyo). Nonspecific protease-free *Clostridium histolyticum* collagenase (form III) from Advance Biofactures Corporation (Lynbrook, NY, U.S.A.). Methyl- ^3H thymidine (specific activity, 20.0 Ci/mmol) and Atomlight (a liquid scintillator) from New England Nuclear (Boston, MA, U.S.A.). Dulbecco's modified Eagle MEM (DE) from GIBCO (Grand Island, NY, U.S.A.). Falcon tissue culture dishes from Becton Dickinson Labware (Oxnard, CA, U.S.A.). Cover slips of Thermanox from LUX Scientific Corporation (Newbury Parks, CA, U.S.A.).

Preparation of Collagen

The dermis of a calf skin was obtained by shaving epidermis with a sharp blade and cut into small pieces in 75% ethanol. The following procedures were carried out aseptically. The dermal pieces were digested by 0.01 g of pepsin per 5 g of the wet tissue in 400 ml of a dilute HCl solution (pH 3) for 3 days at room temperature. The viscous solution thus obtained was passed through a series of filters with different pore size (3, 1, 0.65 and 0.45 μm in this sequence). Pepsin was then inactivated by raising pH of the digestion mixture to 10 and the mixture was kept at 4°C overnight. After adjusting pH of the mixture to 7, the resulting precipitate was collected by centrifugation at 15,000 g for 30 min and dissolved in dilute HCl (pH 3). Collagen was precipitated by neutralizing the solution with 2 N NaOH and solubilized in an HCl solution. This cycle of the precipitation and the solubilization was repeated three times. The final precipitate was dissolved in dilute HCl (pH 3) and used for the present study. Purity of the collagen preparation was checked by SDS-PAGE according to Laemmli (9). The

collagen preparation was treated with nonspecific protease-free bacterial collagenase and subjected to electrophoresis together with untreated preparation (Fig. 1). In the untreated collagen preparation several protein bands were observed, which corresponded to α -chain, β -chain, γ -chain and more polymerized forms of each chain. Treatment of the preparation with pure collagenase eliminated all these protein bands, indicating that the collagen preparation was highly pure.

Cell Culture

Human fibroblasts were obtained from the dermis and cultured as described previously (14, 15). Fibroblasts were grown and maintained in DE

Fig. 1 SDS-PAGE patterns of a calf skin collagen preparation. Acrylamide concentration was 7.5%. The gel was stained by Coomassie Brilliant Blue R-250. Lane a: A collagen preparation digested with bacterial collagenase. One hundred micrograms of the collagen preparation was digested with 100 units of collagenase at pH 7.5 in the presence of 1 mM CaCl_2 for 4 h at 37°C; 3 μg of the digested sample were subjected to electrophoresis. Lane b: 3 units of the bacterial collagenase. Lane c: 3 μg of the collagen preparation. E, band for bacterial collagenase. Arabic numbers, molecular weights of marker proteins. DF, dye front

medium containing 10% FCS, 10 mM NaHCO₃, 20 mM HEPES, 100 units/ml of penicillin and 100 µg/ml of streptomycin in a moist atmosphere of 5% CO₂/95% air at 37°C. The population doubling level (PDL) of the cells used in this study was within 15. Cells in the experiments shown in Fig. 3 through Fig. 8 except Fig. 4 were derived from the same skin explants, but they were in different PDLs at the time of each experiment. Cells shown in Fig. 4 were derived from skin explants from another patient. Cells shown in Table 1 were obtained from the explants which differed from those in Fig. 3 through Fig. 8. Cells were counted with a hemocytometer.

Preparation of Collagen-Coated Dishes

One milliliter of a collagen solution in dilute HCl (pH 3.0) was placed onto 35 mm plastic dishes and dried under a laminar flow of sterile air at 25°C. The solution was dried up within 3 h. Dishes were incubated with 2 ml of the culture medium at 37°C for 1 h prior to use. Fibers were reconstituted by this preincubation, as indicated by the observation that the bottom surface of the dish turned opaque.

Assay of DNA Synthesis

Fibroblasts were incubated in the culture medium containing 1 µCi/ml of [³H]thymidine at 37°C. The isotope incorporation was terminated by adding 1,000 times the excess amount of cold thymidine into the medium. The medium was removed and the cell sheet was washed by PBS containing 50 µM thymidine. Fibroblasts were removed from dishes using either 0.1% trypsin-1 mM EDTA-50 µM thymidine in PBS in case of uncoated dishes or 0.2% bacterial collagenase-1 mM CaCl₂-50 µM thymidine in PBS in case of collagen-coated dishes. Cell pellets were frozen at -20°C until further analyzed. Thawed cells were suspended in 1 ml of water and disrupted for 20 sec at 0°C with a Kontes sonicator (Vine-land, NJ, U.S.A.) tuned to 4 at a maximum power. Following procedures were performed at 0-4°C when not stated otherwise. One milligram of calf thymus DNA was added as a carrier to the test tube containing a sonicated cell suspension. An equal volume of 10% TCA was added to test tubes. A TCA-precipitate was collected by centrifugation at 1,300 g for 15 min and washed 4 times with 5% TCA and boiled for 15 min, then placed at 0°C for 1 h. One half milliliters of the supernatant obtained by centrifugation was mixed with 5 ml of Atomlight and its radioac-

tivity was determined with a liquid scintillation spectrometer (Tracor Analytic Mark III-6811, IL). Ninety-seven percent of the radioactivity in the TCA precipitate was recovered in the supernatant.

Chemical Modification of Collagen

Succinylation of Collagen Collagen was succinylated according to Wang *et al.* (16). Lyophilized collagen powder was dissolved in dilute HCl (pH 3) and pH was adjusted to 9 with NaOH. Succinic anhydride dissolved in acetone was added dropwise to the collagen solution until the quantity of the anhydride reached to 0.4 g per 1 g of dry weight of collagen, maintaining pH at 9-10 by supplying NaOH. The reaction was continued overnight by stirring at room temperature. Succinylated collagen was collected by precipitating it at pH 4.5. Extent of succinylation of ε-NH₂ groups of lysyl residues in collagen was determined by the method of Kakade and Liener (7). Ninety-one percent of the lysyl residues were chemically modified. Helical contents of the modified collagen were estimated measuring the optical rotation at 589 nm with a polarimeter DIP-181 (Japan Spectroscopic Co., Tokyo). A fiber formation of collagen was monitored by measuring the turbidity at 400 nm with a Hitachi-110 spectrophotometer (Hitachi, Tokyo).

Treatment with ethylene oxide gas Freeze-dried collagen powder was gassed with ethylene oxide for 24 h at room temperature. With this treatment, 34% of ε-NH₂ groups of lysyl residues in collagen were chemically modified. They were subjected to hydroxyethylation (11).

Electron microscopic observations

Collagen was coated on a cover slip (30 mm) of Thermanox by the procedure described above. The coated slip was placed in a dish (35 mm) containing 2 ml of PBS and incubated at 37°C for 1 h. The slip which turned opaque was dipped in 2.5% glutaraldehyde, 1% tannic acid in a 0.1 M phosphate buffer (pH 7.4) for 2 h at 4°C. The slip was then post-fixed with 1% OsO₄ in a 0.1 M phosphate buffer (pH 7.4) for 1 h at 4°C. For the transmission electron microscopic (TEM) observations, the fixed slip was dehydrated in a graded series of ethanol and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and examined by a Hitachi HU-12A electron microscope. For the scanning electron microscopic (SEM) observations, the fixed slip was dehydrated in a graded series of ethanol and frac-

Fig. 2 Electron microscopic observations of collagen-coated cover slips. Cover slips of Thermanox were coated with collagen and incubated in PBS at 37°C for 1 h. 1: A view by TEM. $\times 23,000$. 2: A view by TEM. $\times 48,000$. 3: A view by SEM. $\times 30,000$

tured in ethanol cooled in liquid nitrogen. The fractured slips were coated with platinum and viewed through a Hitachi S-700 scanning electron microscope.

RESULTS

Fiber Formation on the Collagen-Coated Dish

The culture medium was placed in a collagen-coated dish. The surface of the dish turned opaque soon when the dish was incubated at 37°C, indicating fiber formation of collagen. To demonstrate the fiber formation directly, a cover slip of Thermanox was coated with collagen and viewed with SEM and TEM. A collagen-coated slip without incubation at 37°C showed no fiber formation. A brief incubation at 37°C resulted in the formation of collagen fibers, which was revealed both by SEM and TEM observations (Fig. 2). The fibers showed a banding pattern with a 65–70 nm periodicity.

DNA Synthesis in Fibroblasts

Time course of [³H]thymidine incorporation into DNA was compared between the cells on the plain and the collagen-coated dishes. Fig. 3

clearly shows that DNA synthetic activity on collagen fibers was remarkably suppressed as compared with that on the plastics. Fibroblasts on a plain dish incorporated the precursor linearly up to 16 h, then the rate of the incorporation declined. In fibroblasts on a coated dish, the incorporation progressed linearly up to 8 h, and the rate was increased 2.5-fold thereafter. This fact suggests the presence of some interactions between cells and collagen fibers or among cells, which influence the kinetics of the isotope incorporation.

To study further on this point, fibroblasts were cultured for different time periods and DNA synthesis was determined within a linear range of the incorporation (Fig. 4). The extent of suppression of DNA synthesis became less remarkable as the culture period became longer. The ratio of DNA synthetic activity on a coated dish to that on a plain dish is 0.20 at day 1 and 0.53 at day 5. This also suggests that fibroblasts interact with collagen fibers and change their properties.

Inhibition of DNA Synthesis in Fibroblasts by Collagen Fibers

DNA synthesis in fibroblasts was determined in

Fig. 3 Time course of DNA synthesis in fibroblasts. One fifth million cells were seeded to dishes (35 mm) with 2 ml of culture medium and maintained for 2 days. Ten microliters of [³H]thymidine (200 μ Ci/ml) was added with a fresh medium. Cells were incubated for the designated periods and processed for the assay of DNA synthesis. Each point represents a single determination. $\circ$ — $\circ$, cells on plain dishes; $\bullet$ — $\bullet$, cells on collagen coated dishes

Fig. 4 DNA synthesis in fibroblasts during the culture on collagen-coated dishes or plain dishes. One tenth million fibroblasts were inoculated in dishes (35 mm) and cultured up to day 7. During the culture, DNA synthesis was determined by replacing with a fresh medium containing [³H]thymidine (1 μ Ci/ml) and incubating for 4 h. Each value is the average of two determinations, the range of which is shown by vertical lines. The open column represents cells on plain dishes, and the closed column cells on collagen coated dishes.

various concentrations of collagen (0.22 to 527 μ g/cm² of growth area) (Fig. 5). Dependence of DNA synthesis on the collagen concentration was clearly demonstrated. No effect was found at 0.22 μ g/cm². DNA synthesis was inhibited in a concentration-dependent manner from 0.66 to 58 μ g/cm², where the effect was maximal.

Fibroblasts take on a specific appearance on collagen fibers which differs greatly from that on a plastic dish (15). As shown in Fig. 6, the colla-

gen-mediated change in the shape was observed only when cells were cultured on the coated dishes with more than 58 μ g collagen/cm², where the maximal inhibition of DNA synthesis was attained. Collagen fibers in the concentration less than 58 μ g/cm² induced the inhibition of DNA synthesis in a dose dependent manner, but did not induce the collagen specific appearance of the cell shape.

DNA Synthesis in Fibroblasts at Different Cell Densities

DNA synthetic activity was determined on both the coated and uncoated dishes with different numbers of cells (Fig. 7). DNA synthesis on the plain dish increased with the cell number up to 2.5×10^5 cells and decreased sharply beyond 2.5×10^5 cells, due to the contact inhibition of cell division. Fibroblasts on collagen fibers showed quite low DNA synthetic activity and contact inhibition of DNA synthesis was not so remarkably observed as on the plastic.

Growth of Fibroblasts

Growth rate of fibroblasts was determined on both plain and coated dishes (Fig. 8). Fibroblasts attached to these two kinds of dishes at the same rate (80% of the inoculated cells). Cell growth on collagen fibers was slower than on the plastic, as was expected from the data on DNA synthesis described above. Doubling time for cells on the plastic was about 24 h and that on the coated dish was 72 h in the growth at day 1 through day 3. There was no difference between the saturation densities on the two kinds of dishes (data not shown).

Abolishment of the Collagen-Induced Effects on Cells by a Chemical Modification of Collagen

Collagen-mediated changes in the shape and growth rate of fibroblasts are abolished by treating collagen with bacterial collagenase or by denaturing collagen (15). The mechanism of the interaction of cells with collagen was studied further, using chemically modified collagen as a substratum. Succinylated collagen dissolved in the culture medium (pH 7.4) was completely denatured within 1.5 h at 37°C, while the extent of denaturation of the ethylene oxide gas-treated collagen was only 8%. Lowering the temperature to 29°C hampered the denaturation (Fig. 9). Eighty percent of the succinylated collagen held a native structure at least for 6 days. No denatura-

Fig. 5 Inhibition of DNA synthesis in fibroblasts by collagen fibers. Varied amounts of collagen (0.22 to 527 $\mu\text{g}/\text{cm}^2$) were coated onto dishes (growth area, 9.1 cm^2). One tenth million fibroblasts were seeded to coated or uncoated dishes. One day after the inoculation, the medium was replaced by a fresh medium containing [^3H]thymidine (1 $\mu\text{Ci}/\text{ml}$) and cells were incubated for 6 h. ●, on collagen-coated dishes; ○, on plain dishes. Each point represents a single determination.

Fig. 6 Micrographs of fibroblasts cultured on collagen-coated dishes. Cells used for the experiment in Fig. 5 were photographed just before adding [^3H]thymidine. A: Cells on plain dishes. B: Cells on the dish coated with 18 $\mu\text{g}/\text{cm}^2$ collagen. C: Cells on the dish coated with 58 $\mu\text{g}/\text{cm}^2$ collagen. D: Cells on the dish coated with 527 $\mu\text{g}/\text{cm}^2$ collagen. $\times 63$

Fig. 7 DNA synthesis in fibroblasts at different cell densities. Fibroblasts, at densities from 1.5×10^4 to $10^6/9.1 \text{ cm}^2$, were cultured for 26 h. DNA synthesis was determined after replacing with a fresh medium containing [^3H]thymidine ($1 \mu\text{Ci/ml}$) and incubating for 15 h. Each point represents a single determination. $\circ$ — $\circ$, cells on plain dishes; $\bullet$ — $\bullet$, cells on collagen-coated dishes

tion was observed for the ethylene oxide gas-treated collagen at this temperature. Ability of fiber formation is shown in Fig. 10 for the succinylated collagen and in Fig. 11 for the ethylene oxide gas-treated collagen. Succinylated collagen completely lost the fiber-forming ability. Fiber formation of ethylene oxide gas-treated collagen was dependent upon the duration of the ethylene oxide gas-treatment. On the basis of these observations, two types of the chemically modified collagens were available, which hold a native helical structure for several days at 29°C , but lose the fiber forming ability.

DNA synthesis in fibroblasts on dishes coated with the two types of the collagens was determined at 29°C (Table 1). In this experiment, gelatin-coated dishes were used as control instead of plain dishes. There is no difference between the DNA synthesis in fibroblasts on a plain dish and on a gelatin-coated dish (15). No suppression of DNA synthesis was found in the fibroblasts on dishes coated with succinylated collagen, and

40% depression was observed on ethylene oxide gas-treated collagen.

DISCUSSIONS

The present study reveals an unexpected effect of collagen on cultured fibroblasts, which is apparently against the generally accepted role of collagen in the proliferation of cells *in vitro*. Our experiments demonstrate clearly and reproducibly that native collagen is inferior as a substratum for fibroblasts to the tissue culture plastic and the denatured collagen with respect to promotion of DNA synthesis and proliferation of the cell.

We have demonstrated that fibroblasts take on a specific shape with abundant cytoplasmic protrusions and their DNA synthesis is remarkably reduced when they contact native collagen, but not when they contact denatured collagen (15). To our knowledge, among many papers that described the positive effects on various kinds of

Fig. 8 Growth of fibroblasts on collagen-coated (●-●) and plain (○-○) dishes. The number of cells was plotted against days in culture.

Fig. 9 Changes in helical contents of the chemically modified collagen. The ethylene oxide gas-treated (○-○) or the succinylated (●-●) collagen was dissolved in Eagle's MEM (pH 7.4) and incubated at 29°C for 6 days. At days indicated, helical contents of collagen were determined as described in the text. Each point represents a single determination.

Fig. 10 Inability of succinylated collagen to form fibers. The succinylated collagen was dissolved at 2 mg/ml in Eagle's MEM containing 10% FCS. Collagen was gelated at 29°C. At time points indicated, the extent of gelation was determined by measuring the turbidity at 400 nm. ○-○, succinylated collagen; ●-●, native collagen

Fig. 11 Fiber formation of ethylene oxide gas-treated collagen. Collagen was gassed with ethylene oxide for varied lengths of time. The treated collagen was dissolved at 2 mg/ml in Eagle's MEM containing 10% FCS and gelated at 37°C. At time points indicated, gelation was determined by measuring the turbidity at 400 nm. Untreated collagen, ○-○. Collagen treated for 0.7 h, ●-●; for 1.33 h, ▲-▲; for 3.75 h, ■-■; for 8 h, □-□; for 24 h, ▽-▽

Table 1 DNA Synthesis in Fibroblasts on the Chemically Modified Collagen

Substrata	DPM/Dish	% of 'Gelatin'
Native collagen	130,281	16
	106,686	
Gelatin	704,791	100
	780,665	
Succinylated collagen	720,603	101
	773,621	
Ethylene oxide gas-treated collagen	441,711	61
	462,758	

Dishes (35 mm) were coated with 1 ml of 0.36% collagen, gelatin, succinylated collagen or ethylene oxide gas-treated collagen. One fifth million fibroblasts were seeded to each of these dishes and cultured at 29°C for 42 h. DNA synthesis was determined after replacing the medium with a fresh medium containing 1 μ Ci/ml [3 H]thymidine and incubating for 77 h at 29°C. Each value represents a single determination.

cells only one paper by Elsdale and Bard (2) reported the negative effect of collagen on DNA synthesis in fibroblasts. They found that DNA synthesis in fibroblasts in contact with collagen was reduced to 50% of that on tissue culture plastic.

Some impurities in the collagen preparation used in the present study might be responsible for the inhibition of DNA synthesis in the cell. However, this possibility is unlikely, because the collagen preparation seems to be pure enough (Fig. 1). Treatment of the collagen preparation with collagenase abolishes the inhibitory action of collagen fibers on DNA synthesis in fibroblasts (15), which again supports the view that collagen is an active principle.

DNA synthesis in fibroblasts is highly dependent upon the quantity of collagen which the cell contacts (Fig. 5). Minimum effective quantity of collagen was around 0.7 μ g/cm² when fibroblasts were seeded at 1.1×10^4 cells/cm², indicating that 60 pg of collagen/cell is effective in suppressing DNA synthesis. Kleinman *et al.* (8) reported that attachment of Chinese hamster ovary cells to a plastic dish is enhanced by coating a dish with as low as 1 ng/cm² of collagen.

According to Grinnell (3), the process of cell adhesion progresses as consecutive four stages; adsorption, contact, attachment and spreading. In cells used in the present study, collagen has no effect on the initial three stages (Fig. 8). However we noticed that collagen caused a significant delay in cell spreading.

DNA synthesis in fibroblasts on collagen

fibers is biphasic (Fig. 3). The extent of suppression of DNA synthesis on collagen decreased as the duration of culture on the collagen-coated dish increased (Fig. 4). The suppression is much less at day 5 than at day 1. All these observations suggest that there exists some interactions between cells and collagen, which cause changes in the properties of collagen.

The cell-to-cell interaction on a coated dish appears to differ from that on an uncoated dish (Fig. 7). Contact inhibition of cell proliferation was clearly manifested on a plain dish, but not so clearly on a collagen-coated dish. For example, DNA synthesis at 10^6 cells was about 10% of that at 2.5×10^5 cells in case of plain dishes, whereas about 25% in case of collagen-coated dishes.

What is a physiological meaning of the 'collagen-dependent suppression of DNA synthesis'? Fibroblasts are believed to attach to collagen fibers complexed with proteoglycans and glycoproteins, including fibronectin. This complex is likely to be a natural substratum for fibroblasts. Proliferation of fibroblasts *in situ* can be easily and delicately controlled by activators or inhibitors working under physiological and pathological conditions. Fibroblasts in healthy adult tissues *in vivo* are quiescent and remain in the G₀ stage (6, 12). We suggest that the interaction between fibroblasts and collagen fibers is one of the reasons for their quiescence *in vivo*.

Biological functions of collagen, such as control of the specific cellular shape or regulation of DNA synthesis described in this paper, are manifested only by a native and polymeric form of collagen (Table 1; 15). Observations made by Tomasek *et al.* (13) support our interpretation. They demonstrated that embryonic and fibroblast fibroblasts respond to hydrated collagen by becoming elongated, whereas these cells are highly flattened on plastic dishes. The collagen-mediated change in the cell shape was not observed when the fibroblasts were in contact with soluble collagen.

It is probable that an ϵ -amino group of lysyl residues is essential for the interaction of collagen with cells, because the extent of suppression of DNA synthesis appears to be proportional to the extent of blockade of the ϵ -amino group of lysyl residues (Table 1).

The authors express their thanks to Dr T. Miyata for his advice on using the chemically modified collagens as substrata for culture of fibroblasts. This work was supported in part by a grant-in-aid for special project research from the Ministry of Education, Science and Culture, Japan (No. 59105003), by a grant from the Ministry of Health and Welfare, Japan, and by a grant

for Special Research Projects of Kitasato University School of Medicine (No. 83502).

Received for publication 9 January 1985

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