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Intercellular Accumulation of Type V Collagen Fibrils in Accordance with Cell Aggregation

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We reported previously that human fibroblasts form clumps when cultured on a dish coated with reconstituted type V collagen fibrils. Essentially all the type V collagen fibrils, initially coated on the dish, were recovered in the cell clumps that had eventually formed during the culture. We interpreted that type V collagen fibrils adhere to cells more strongly than to the dish and are detached by cell movements. In this study, type V collagen was suspended with fibroblasts to examine the fate of the type V collagen fibrils and to determine whether the fibrils affect the behaviour of the cells directly adherent to the dish. The added type V collagen accumulated in the intercellular space concomitantly with the local aggregation of fibroblasts. scanning electron microscope examination indicated that type V collagen fibrils were found in the vicinity of cells in cultures without ascorbic acid where essentially no collagen secretion takes place. These results indicate that type V collagen forms fibrils and the fibrils are accumulated in the intercellular spaces. The accumulated type V collagen fibrils work as a cementing material for cell clump formation. This phenomenon is discussed in relation to the possible involvement of type V collagen fibrils in tissue organization.

Key words: accumulation of collagen fibrils, cell cementing, cell–collagen interaction, clump formation, type V collagen fibrils.

Abbreviations: PBS, calcium-free magnesium-free phosphate buffered saline; FITC, fluorescein isothiocyanate; SEM, scanning electron microscope.

Collagens are the major components of the extracellular matrix, and they form the skeletal structure or the framework of various organs. Type V collagen as well as types I, II, III and XI collagen are members of the fibrillar collagen family, comprising the collagen fibrils in connective tissues (*1–3*). Type V collagen is widely distributed in many tissues, including the skin, placenta, skeletal muscle and cornea, forming fine fibrils or limiting the fibrillar diameter (*4–8*). Mutant mice deficient in the procollagen α1(V) chain die in early embryogenesis (*9*). Mice with structurally abnormal α2(V) collagen chains have poor survival rates (*10*). They exhibit disorganized collagen fibrils in the skin and eye tissues. These observations imply that type V collagen plays a central role in physiological collagen fibrillogenesis.

Studies of cell cultures with collagen aggregates, which are the major forms of collagenous proteins *in vivo*, demonstrate that the effects of collagen aggregates on cell attachment, migration, differentiation and proliferation are distinct from those of monomeric collagen when these proteins were used as cell culture substrates (*11–14*). The differential effects of different collagen types could be in part due to the distinct adhesion molecules of cells (*15–17*). Type V collagen molecules are mostly colocalized with type I collagen molecules as hybrid collagen fibrils (*5*, *18–20*). Linsenmayer and colleagues (*21–23*) reported that the triple helical domain of type V collagen is not localized on the surface of the fibrils. However, in our anatomical and biochemical studies (*18*, *24*), type V collagen molecules in collagen fibrils were found to be located on the surfaces of collagen fibrils, and thus they are often in direct contact with cells *in vivo*. Furthermore, at a particular stage of tissue development or at the initial stage of wound healing when the production of type I collagen is not yet sufficient for deposition, type V collagen molecules alone may comprise collagen fibrils. The examination of the effects of type V collagen fibrils on cultured cells may provide us with clues to understanding the biological functions of type V collagen in affecting cell behaviours.

It has been reported that type V collagen molecules act as anti-adhesive agents against cultured cells (*25*, *26*): Vascular endothelial cells in particular tend to detach from dishes coated with type V collagen molecules (*27*, *28*), suggesting that type V collagen prevents cell adhesion to the culture dish. We have proposed that the

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strong adhesion of type V collagen to cells causes apparent detachment of the cells from the dish. The hypothesis is deduced from the finding that human fetal lung fibroblasts formed cell clumps when the cells were cultured on a dish coated with reconstituted type V collagen fibrils (*29*). The following possible mechanism is postulated for this finding. Seeded cells initially adhere to type V collagen fibrils on the culture dish. Changes in cell morphology, including the protrusion and retraction of cell processes, accompany the detachment of type V collagen fibrils from the dish, since the adherence of these fibrils to the dish is weaker than that to the cells and the fibrils cannot resist the retracting force exerted by the cells. Thus, the movements of cells with adherent type V collagen fibrils will result in the condensation of the fibrils in the intercellular space, resulting in the cementing of cells to one another.

In the present study, the cell-adherent property of type V collagen fibrils is directly examined by investigating the fate of the type V collagen added exogenously to the culture medium. Electron microscopic analysis shows that type V collagen fibrils reconstituted solely from the major triple helical domain of pepsin-treated molecules are straight and uniform in diameter and without any branches (*30*). Therefore, exogenously added type V collagens are expected to form straight and separate fibrils that directly interact with cultured cells.

MATERIALS AND METHODS

Cells and Cell Culture—Human diploid fetal lung fibroblasts, TIG-1, established at the Tokyo Metropolitan Institute of Gerontology were provided by Dr Kiyotaka Yamamoto (*31*). Human dermal fibroblasts, HF-18, were provided by Dr Toshio Nishiyama of Shiseido Research Center, Yokohama (*32*). Human neonatal dermal fibroblasts, HDFneo, were purchased from Sanko Junyaku Co., Ltd (Tokyo, Japan).

These cells were cultured in Dulbecco's modified Eagle's medium (Sigma Chemical, MO, USA) containing 10% fetal bovine serum (Cansera International Inc., Ontario, Canada), 50 U/ml penicillin G and 50 μg/ml streptomycin sulphate (GIBCO BRL, Invitrogen Corp., Carlsbad, CA). The cells were maintained at 37°C under a humidified atmosphere of 5% CO_2 and 95% air on 60 mm tissue culture dishes (BD Falcon, BD, Franklin Lakes, NJ). When the cells reached confluence, they were removed from the dishes by treatment with 0.025% trypsin (BD Difco, BD), 0.02% ethylenediamine tetra-acetic acid in calcium-free magnesium-free phosphate buffered saline (PBS; Nissui Pharmaceutical, Japan) and subcultured at a 1 : 4 split ratio.

Unless otherwise indicated, these cells were plated at a density of 1.0×10^4 cells/cm^2 into 24-well plates (BD Falcon), 4-well plates or an 8-well chamber slide (Nalge Nunc International KK, Tokyo, Japan), and maintained in the above medium to which type V collagen (20 μg/ml) was added. The medium was replaced every 3 days. Cell morphology was observed with a phase contrast microscope (Model DMIRB; Leica Microsystems Co., Tokyo, Japan) at 100× magnification.

Type V Collagen—All the following procedures were carried out at 4°C except for column chromatography that was performed at room temperature. Type V collagen was essentially prepared from bovine corneas (purchased from Tokyo Shibaura Zoki, Tokyo, Japan) according to the method described by Miller and Rhodes (*33*). Briefly, bovine cornea homogenates were suspended in 0.5 M acetic acid containing 1 mg/ml pepsin (Worthington Biochemical Corporation, NJ, USA); the type V collagen liberated was separated by salt fractionations in both acidic and neutral solutions and was then purified by heparin column chromatography as described previously (*34*). In brief, an appropriate amount of the collagen solution in 0.5 M acetic acid was dialysed against PBS containing 2 M urea and 0.07 M NaCl before the solution was applied on a heparin-Sepharose (Amersham Biosciences, Uppsala, Sweden) column. After the column was washed with PBS containing 2 M urea and 0.07 M NaCl, the bound protein was eluted with PBS containing 2 M urea and 0.5 M NaCl. The eluted solution was dialysed against 0.5 M acetic acid and then type V collagen was precipitated by salting out. The precipitates were treated with 70% ethanol for sterilization. After the type V collagen was dissolved in 0.5 M acetic acid, the solution was dialysed against 1 mM HCl and stored at 4°C before use. Protein quality was determined by SDS–PAGE for any contaminates such as type I and type VI collagens. Protein concentrations were estimated from the dry weight per fixed volume of the lyophilized material.

Fluorescein Isothiocyanate-pre-labelled Type V Collagen—Fluorescein isothiocyanate (FITC)-pre-labelled type V collagen was prepared as described previously (*29*). In brief, a type V collagen solution in 1 mM HCl was mixed with an equal volume of 0.5 M $NaHCO_3$–Na_2CO_3 buffer, pH 9.5. An amount of FITC (Sigma Chemical) equal to 3.5% of the dry weight of type V collagen was added to the collagen solution in carbonate buffer and then incubated with stirring at room temperature for 3 h in the dark. The solution was neutralized with 6 M HCl at 4°C and treated with 70% ethanol for precipitation and sterilization of the FITC-conjugated collagen. This FITC-labelled collagen was dissolved in 0.5 M acetic acid, and the solution was then dialysed against 1 mM HCl at 4°C. Confirmation of FITC covalent binding to type V collagen α chains was carried out by fluorescence image analysis of the SDS–PAGE pattern of FITC-labelled type V collagen. TIG-1 cells cultured with FITC-pre-labelled type V collagen were examined by fluorescence and phase contrast microscopy.

Cell Proliferation Assay—Relative cell numbers were determined by using a cell proliferation assay system, namely, Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan), according to the manual provided by the manufacturer. The TIG-1 cells were plated at a density of 1.0×10^3 cells/well into 96-well plates (BD Falcon) and maintained in the above medium containing PBS, type I collagen (10 μg/ml), type V collagen (20 μg/ml), or heat denatured type V collagen (20 μg/ml). The medium was replaced every 3 days. The reagent was added to the wells and then the cultured cells were incubated in the wells for 60 min before the absorbance at 450 nm and 600 nm were recorded with a Synergy HT (BioTek Instruments, Winooski, VT). This system uses

tetrazolium salt (WST-8) for counting cell numbers. The tetrazolium salt is cleaved to soluble formazan dye by succinate-tetrazolium reductase, which exists in the mitochondrial respiratory chain. The quantity of formazan dye is related directly to the number of metabolically active cells. The absorbance of 450 nm is formazan dye and the absorbance of 600 nm is reference.

Fluorescence Microscopy—The cells were fixed with 3.7% paraformaldehyde in 100 mM phosphate buffer (pH 7.4) for 30 min at room temperature and then permeabilized by treatment with 0.5% Triton-X100 in PBS for 10 min at room temperature. They were pre-incubated with 3% bovine serum albumin in PBS for 90 min at room temperature. After washing with PBS, the cell culture specimens were treated with a Texas Red–phalloidin (Molecular Probes, OR, USA). They were examined with a fluorescence microscope or a confocal laser scanning microscope (TCS-SP, Leica Microsystems AG, Wetzlar, Germany).

Scanning Electron Microscope—The fetal lung fibroblasts TIG-1 were fixed with half-strength Karnovsky's fixative for 1 h at room temperature and post-fixed with 1% OsO_4. The fixed cells were dehydrated with a graded series of ethanol up to 95% and infiltrated with tertial butyl alcohol with three changes. They were then freeze-dried with a freeze-dryer (Eiko Type ID-2, Eiko Co. Ltd, Tokyo, Japan) overnight at −20°C and coated with gold using a sputter coater (Eiko Type VX-10A, Eiko Co. Ltd, Tokyo). Finally, the specimens were observed with an scanning electron microscope (SEM; S-4500, Hitachi Co. Ltd, Tokyo, Japan).

Time-lapse Observation—HDFneo cells were cultured on a dish coated with type V collagen fibrils. Cell behaviours were recorded by time-lapse video microscopy. Briefly, dishes coated with type V collagen fibrils were prepared by mixing 9 volumes of the collagen solution in 1 mM HCl with 1 volume of 200 mM phosphate buffer containing 1.5 M NaCl, pH 7.3, at 4°C and placing the neutralized solution (with a final concentration of 100 μg/ml) into 35 mm dishes. After the solution was maintained at 37°C for 24 h to allow fibrillar aggregates to form and adsorb to the dish surface, the wells were washed twice with PBS. The cell suspensions prepared as described above were then plated on each substrate (2×10^4 cells/cm^2) and were cultured with the above culture medium at 37°C under a humidified atmosphere of 5% CO_2 and 95% air on a microscope stage (Stage top incubator INU series, Tokai Hit Co., Ltd, Shizuoka, Japan). The cells were cultured for 7 days, and the culture medium was replaced every 3 days. Cell morphology was observed with a phase contrast microscope (Model DMIRB, Leica Microsystems Co., Tokyo, Japan), and the images were obtained by a charge-coupled device camera (ORCA, Hamamatsu Photonics, Hamamatsu, Japan). The images were sequentially imported by Lumina Vision software (Mitani Corporation, Fukui, Japan) at intervals of 4 min.

RESULTS

Culture of TIG-1 Cells with FITC-pre-labelled Type V Collagen—Human fetal lung fibroblast, TIG-1, cells were cultured with exogenously added type V collagen and then suspended into the culture medium. Since the medium did not contain ascorbate, the minimum effect of *de novo* production of collagens was expected (*35*, *36*). The cells adhered to and spread on the dish initially as observed in the case of the culture without type V collagen (data not shown). However, cell proliferation was delayed as compared with that in the culture without type V collagen (Fig. 1). The cells proliferated normally in the culture with type I collagen or with denatured type V collagen (Fig. 1). Moreover, cell aggregation occurred in the culture with type V collagen (Fig. 2). The cells were piled up on each other exhibiting a 'hill and valley'-like pattern at a late stage of culture (Fig. 2). The TIG-1 cells tended to lose adherence to the dish when type V collagen was present in the culture medium. Some portion of the cell aggregates detached from the dish particularly at the time of medium exchange due to the friction force exerted by medium suction and addition. The result showed that exogenous type V collagen facilitated cell detachment from the culture dish and also functioned as a cell glue.

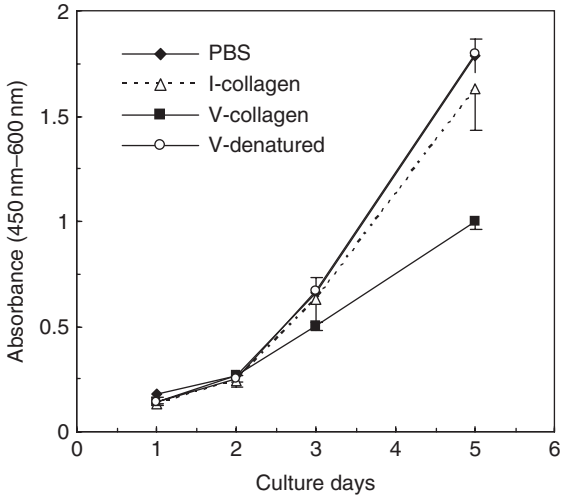

Fig. 1. **Effect of type V collagen on the proliferation of TIG-1 cells in culture.** The cells were cultured with PBS (closed diamond and solid line), type I collagen (open triangle and dashed line), type V collagen (closed square and solid line), or denatured type V collagen (open circle and solid line). The cells were seeded on 96-well culture plates (1.0×10^3 cells/well) on day 0.The proliferation of the TIG-1 cells cultured with type V collagen was delayed. The ordinate, i.e. absorbance (450–600 nm), represents the relative cell numbers (see MATERIALS AND METHODS section for more details). Values are shown as mean ± SD of experiments in triplicates.

The cells also aggregated when they were cultured with FITC-labelled type V collagen (Fig. 2), indicating that the labelling of type V collagen with FITC did not result in any essential difference in its effect on cell behaviour. The fate of the FITC-labelled type V collagen was monitored by fluorescence microscopy. At the beginning of the culture with FITC-labelled type V collagen, the distribution of fluorescence was homogeneous with weak intensity. Within 6 h of culturing, condensed fluorescence spots became apparent, while the surface of the

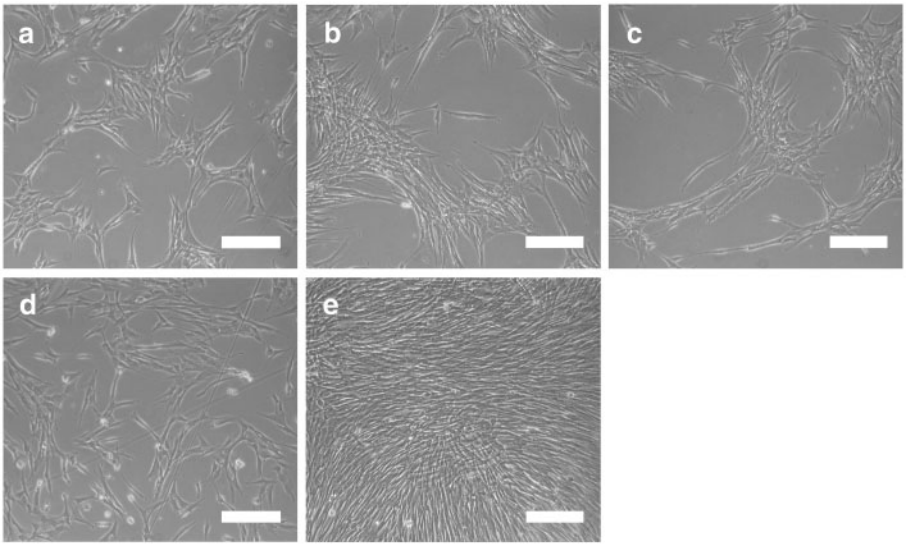

Fig. 2. **Phase contrast micrographs of TIG-1 cells cultured with type V collagen.** Culture for 1 day (a, d) or 3 days (b, c, e). TIG-1 cells cultured with type V collagen (a, b), FITC-labelled type V collagen (c) and PBS (d, e). Aggregated cells are piled up in (a), (b) and (c), but scarcely in (d) and (e). Bar: 200 μm.

dish showed only weak fluorescence where cells were absent (Fig. 3A). The number and size of the condensed fluorescent spots increased in a time-dependent manner. Fibrous fluorescence was seen on the cell surface by day 3 (Fig. 3A). These results suggest that the condensed fluorescence spots represent the enriched type V collagen exogenously added, whereas the weak fluorescence on the dish surface represents the FITC-labelled type V collagen that are not captured by the cells. It is thus most likely that some of the type V collagen in a fibril form adhered to the TIG-1 cells and gradually condensed onto the cells.

Condensation of Type V Collagen—The cell aggregation concomitant with the condensation of type V collagen fibrils onto the cell surfaces was also seen in the culture with serum-free medium (Fig. 3B). The cells secrete some factors for the condensation of type V collagen fibrils. The condensation of type V collagen fibrils onto the cell surfaces was less pronounced in the serum-free medium, suggesting that serum also contains some key factors for the condensation of type V collagen fibrils on cell peripheries.

When heat-denatured FITC-labelled type V collagen was used in place of native type V collagen, the cells did not aggregate (Fig. 3B). Cell proliferation with denatured type V collagen was as high as that without type V collagen (Figs 1 and 3B). Denatured type V collagen does not affect cell behaviour, even though the polypeptides adhere to the cells. Furthermore, no fluorescent spots were observed (Fig. 3B). The results indicate that the triple helical conformation, which is required for fibril formation, is crucial for the interactions of type V collagen with cells. Type V collagen fibrils function as cell glue materials.

Condensation of Type V Collagen on Human Dermal Fibroblasts—TIG-1 cells are human fetal lung fibroblasts that produce type IV collagen in addition to type I, type III and type V fibrillar collagens (*37*). Using ascorbic acid-free culture medium, although the secretion of fibrillar collagen was minimized, non-triple helical chains of type IV collagen are secreted into the culture medium (*38*). In order to examine whether the phenomenon observed for TIG-1 cells can be extended to mature human dermal fibroblasts, HF-18 cells that do not secret type IV collagen were cultured with FITC-labelled type V collagen. HF-18 cells aggregated and also exhibited condensed FITC spots (Fig. 3C). Many FITC spots were observed in the cell layer (Fig. 3C), while some spots were also found on the cell peripheries, suggesting that type V collagen fibrils have a strong affinity for the cells. It is concluded that human fibroblasts accumulate the exogenously added type V collagen, and the accumulated type V collagen fibrils are responsible for cell aggregation and work as cell glue matrix.

Distribution of Accumulated Type V Collagen—The distribution of condensed type V collagen was analysed by confocal laser scanning microscopy. The FITC spots were enriched in the intercellular space but were sparsely observed on or underneath the cells cultured for 3 days (Fig. 4). Thus, type V collagen fibrils are well condensed in the intercellular spaces.

The supramolecular structure of condensed type V collagen was examined with an SEM for the TIG-1 cells cultured with type V collagen for 4 days (Fig. 5). The secretion of collagens was minimized by using an ascorbic acid-free culture medium since ascorbic acid is essential for the production of sufficient collagen molecules for fibril deposition (*35*, *36*).

A number of collagen fibrils were observed on and around the cultured TIG-1 cells when the culture medium was exogenously supplemented with type V collagen (Fig. 5). On the contrary, no fibrils were seen on and around the cells when the cells were cultured without exogenous type V collagen (Fig. 5). These fibrils

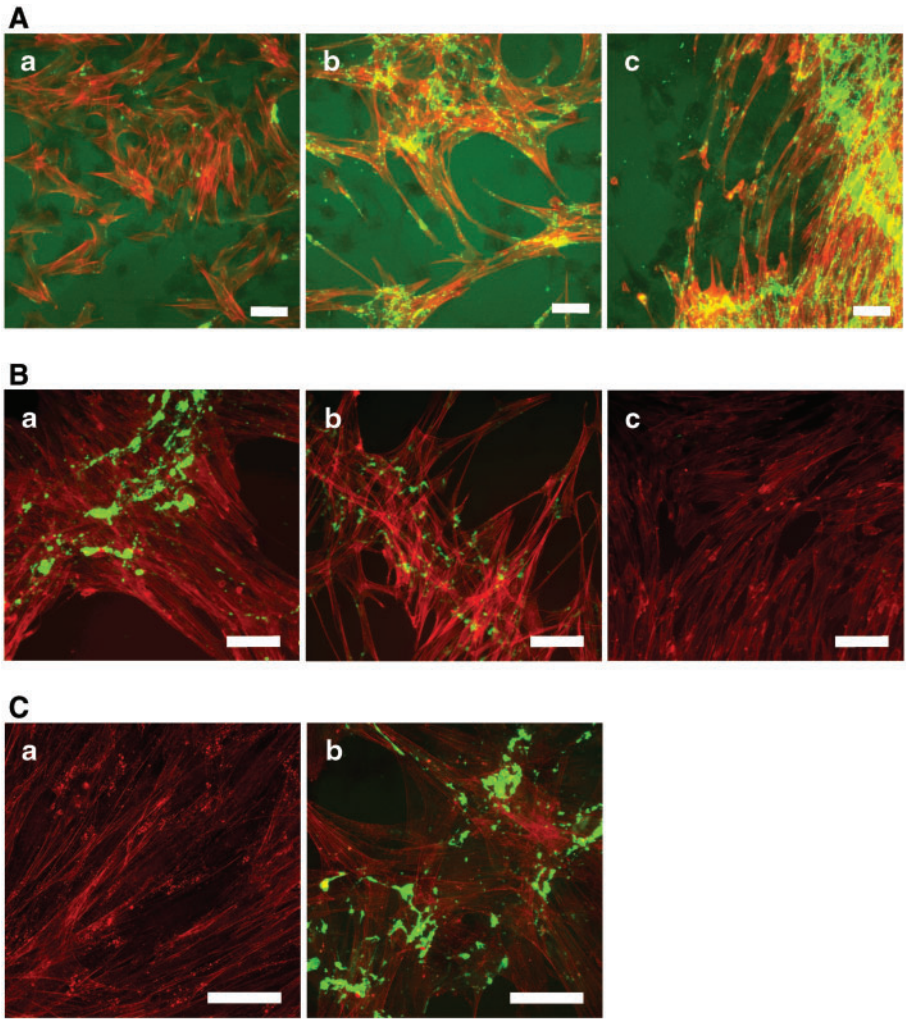

Fig. 3. **Localization of added FITC-labelled type V collagen.** (A) TIG-1 cells were cultured with FITC-labelled type V collagen for 6 h (a), 32 h (b) or 3 days (c). Green colour reflects FITC-labelled type V collagen, whereas red colour is from the actin filaments stained with Texas red-phalloidin. Green fluorescence spots gradually increased in number as well as in size as the culture time was prolonged. Bar: 100 μm. (B) TIG-1 cells were cultured with (a) or without (b) serum in the presence of FITC-labelled type V collagen for 3 days. The cells cultured for 3 days with native FITC-labelled type V collagen (a) or denatured FITC-labelled type V collagen (c) with serum. Green, FITC-labelled type V collagen. Red, actin filaments. Bar: 100 μm. (C) HF-18 cells (human dermal fibroblasts) were cultured with PBS (a) or FITC-labelled type V collagen (b) for 3 days. Green, FITC-labelled type V collagen. Red, actin filaments. FITC-labelled type V collagen was condensed into cell peripheries. Bar: 100 μm.

with an axial banding pattern typically found in collagen fibrils were seen on the surface of the cells as well as in the intercellular spaces (Fig. 5). Although it is difficult to measure a precise repeating length of the banding pattern from the SEM image, the rough estimate of the length is about 50 nm. Moreover, thinner fibrils that appear to attach directly to the cells are ~50 nm in diameter, and thicker fibrils that may be bundles of thin fibrils are found in the intercellular spaces (Fig. 5). The thinner fibrils are reminiscent of *in vitro* reconstituted type V collagen fibrils (*30*). From the light microscopic result that the exogenously added type V collagen was clearly condensed in cell layer (Fig. 3A), it is most likely that most of the observed collagen-like fibrils, if not all, are derived from the added type V collagen fibrils. Therefore, we concluded that the added type V collagen molecules formed fibrils in the culture medium and the fibrils adhering to the cell surfaces were condensed forms of the exogenously added type V collagen fibrils.

Time Lapse Observation of Human Fibroblasts on Type V Collagen Fibrils—The behaviour of fibroblast cells on the type V collagen fibrils were essentially the same among the cell types examined so far in a point of view that cells form the cellular aggregates. The differences between cell types were the extent of the aggregates or the time required for the clump formation. Human neonatal dermal fibroblast, HDFneo, is one of such cells and was successfully observed about its dynamic movement resulting in the cell aggregation-induced type V collagen fibrils. HDFneo cells were cultured on a dish coated with type V collagen fibrils and recorded by time lapse photography. HDFneo cells grew on the dish, and the cells gradually aggregated with each other on the type V collagen fibrils (Fig. 6A and Movie 1, 2). The cell-free

spaces were enlarged during cell aggregation. Eventually, the cell aggregates detached from the dish surface and formed a cell clump (Fig. 6B and Movie 3). The cell detachment is dynamic phenomenon and it looks like shrinking of stretched rubber. These results suggested that cell movement exerted driving forces towards cell aggregation. This observation is consistent with the hypothesis that cell aggregation in the form of clumps by culture with type V collagen fibrils is caused by cell movement together with the adherence property of type V collagen fibrils.

DISCUSSION

At first, we discuss about the relation and difference between our previous study and the present study. In the previous study, using culture dish coating with type V collagen, we show that type V collagen fibrils adhere to fibroblasts and interpret the mechanism of the cell clump formation by assuming that type V collagen fibrils adhere to the fibroblasts more strongly than to the dish (*29*). In the present study, by adding type V collagen fibrils to cultured fibroblasts, we observed the dynamical interaction between type V collagen fibrils and fibroblasts. Since fibroblasts secrete some extracellular matrices including type I collagen, we cultured the cells using ascorbic acid-free culture medium to minimize secretion of type I collagen (*35*, *36*). Adding type V collagen caused gradual cell aggregation and the cell aggregations easily detached from the culture dish. Direct adherence of fibroblasts to the surface of culture dish will be the reason for the low affects of type V collagen fibrils to the fibroblasts. Thereby, we can observe the process of the clump formation of fibroblasts by culturing with type V collagen fibrils. The mechanism of and the prerequisite for the clump formation caused by type V collagen fibrils become clear in the present study.

A mechanism is schematically shown in Fig. 7 for the aggregation and clump formation of cells involving concomitant accumulation and condensation of type V collagen fibrils into the intercellular spaces. Initially, these fibrils adhere to the cell surface through their receptors. The adherent type V collagen fibrils move over the cell surface. These fibrils are interlinked and bundled together on the cell surface (Fig. 7A). Serum factors and some factors secreted in the cell peripheries accelerate the bundling of the type V collagen fibrils (Fig. 3B). The interlinked and bundled type V collagen fibrils correspond to the spots of FITC on the cell surface (Fig. 3A). The type V collagen fibrils on the cell surface support

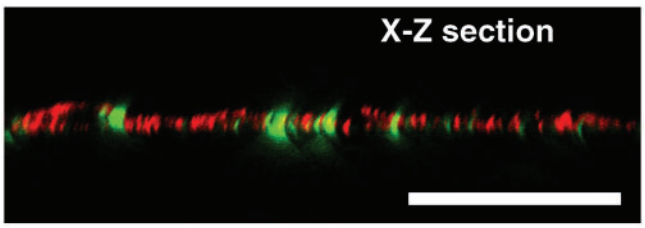

Fig. 4. **Tomographic localization of FITC-labelled type V collagen.** TIG-1 cells were cultured with FITC-labelled type V collagen for 3 days. The deposition of FITC-labelled type V collagen was observed by confocal laser scanning microscopy for 3D reconstruction. Green, FITC-labelled type V collagen. Red, actin filaments. The image of an *X–Z* section is shown. The FITC spots are mainly observed in the intercellular spaces; F-actin labelling represents the intracellular spaces. Bar: 50 μm.

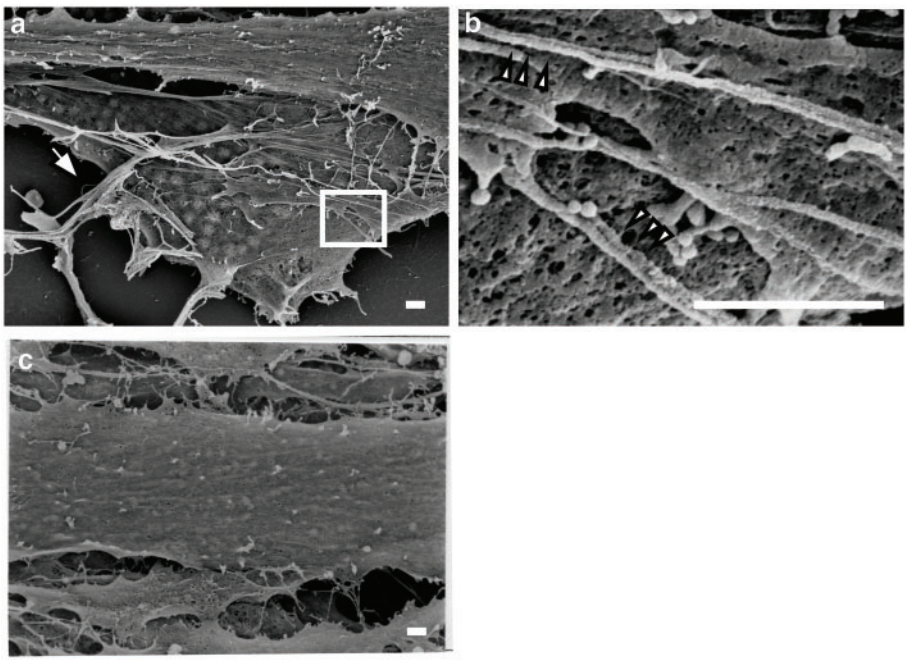

Fig. 5. **Scanning electron micrographs of TIG-1 cells cultured with FITC-labelled type V collagen.** TIG-1 cells cultured with (a, b) or without (PBS only) FITC-labelled type V collagen (c) for 3 days. Fig. 5 (b) shows a high magnification of the boxed region of Fig. 5 (a). Arrowheads, banding pattern of fibrils; arrow, bundled collagen fibrils. Bar: 1.0 μm.

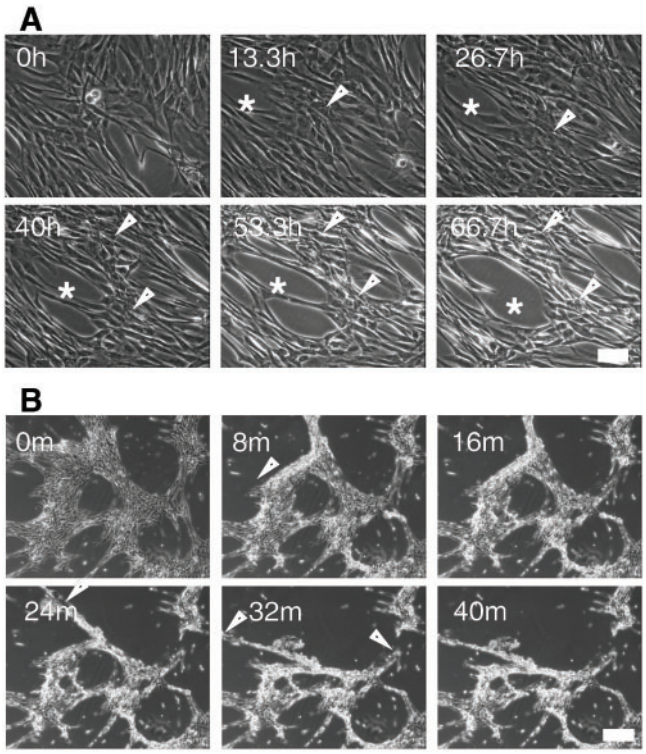

Fig. 6. **Time lapse images of HDFneo cells cultured on the type V collagen fibrils.** HDFneo cells were cultured on the dish coated with the type V collagen fibrils for 7 days. (A) A series of phase contrast images of HDFneo cells cultured from 4 to 6 days. The images were obtained every 13.3 h at the same position. The cells moved and gradually aggregated with each other (arrowheads). Cell-free spaces (asterisks) formed during the process of aggregation are enlarged. Bar: 100 μm. Movie 1 is constructed from the series of the images of the cells cultured from 4 to 6 days. Movie 2 is constructed from the series of the images of HDFneo cells cultured from 6 to 7 days. (B) A series of phase contrast images of HDFneo cells cultured at 7 days and obtained every 8 min at the same position shows the moment of the detachment of the cells from the culture dish. These cell aggregates detached spontaneously (arrowheads). Cell detachment looks like the shrinking of stretched rubber. Bar: 200 μm. Movie 3 is constructed from the series of the images for 160 min.

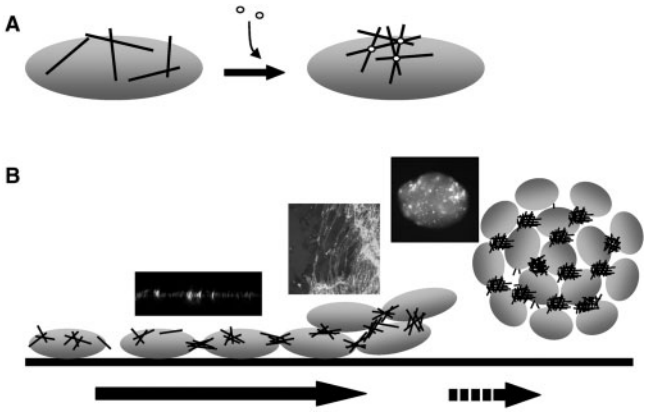

Fig. 7. **A schematic drawing to show a mechanism of cell clump formation together with the accumulation and condensation of type V collagen fibrils into the intercellular spaces.** Type V collagen fibrils are represented by black lines. (A) Type V collagen fibrils adhere to the cell surface, and the adherent collagen fibrils move on the cell surface. When substances (open circle) that can bind type V collagen fibrils are secreted on cell peripheries, the adherent type V collagen fibrils are interlinked with each other. (B) Cells encounter each other as they move. Interlinked and bundled type V collagen fibrils mediate connections between these cells. The size and density of condensed type V collagen fibrils increase, particularly in the intercellular space accommodating the fibrils. The strong affinity of type V collagen fibrils with the cells as well as with the substances that combine type V collagen fibrils result in the cementing of cells. Exogenously added type V collagen eventually condenses on cell aggregates, functioning as cell cement in the process.

cell–cell interaction. The fibrils between two adjacent cells interlink with each other. The interlinking type V collagen fibrils in turn mediate the cementing of cells to one another (Fig. 7B). Consequently, the cells gradually aggregate (Figs 2 and 6A). Eventually, the size and density of the accumulated type V collagen fibrils increase, particularly in the intercellular region (Fig. 4). The aggregated cells are cemented with type V collagen fibrils (*29*). Cell movement is an important factor that contributes to the acceleration of cell aggregation that leads to clump formation (Fig. 6). Thus, exogenously added type V collagen fibrils mediate the intercellular connections between fibroblasts.

What is a possible mechanism for the condensation of type V collagen fibrils onto the cell surface and eventually into the intercellular space? When fibroblasts are cultured in type I collagen gel, collagen fibrils are condensed in the extracellular periphery of the cells (*39*). In a previous report, we discussed that cell movements, including rotation, induce the denser packing of collagen fibrils that are adherent to each other. The packing process of type I collagen fibrils involves the forces of retraction that are caused by cell motility, including the protrusion and retraction of cell processes (*40*). It is likely that the type V collagen exogenously added formed fibrils that were suspended in the culture medium or some portion sedimented onto the dish. The added type V collagen fibrils are freely distributed in space of the culture medium. In that sense, the type V collagen fibrils exist three-dimensionally as the type I collagen gel. The latter can move much less freely in a gel form. The condensation of type V collagen fibrils into the intercellular space can be accounted for by mechanism similar to that responsible for the condensation of type I collagen fibrils into the vicinity of cells. In short, type V collagen fibrils will be moved, accumulated and condensed by the mechanical forces generated by cell motility through yet unidentified cell surface mediator(s).

However, cell motility alone cannot explain the accumulation and condensation of the fibrils made up of type V collagen. Interaction between type V collagen fibrils is essential for fibril bundling. In view of the dispersed distribution of the reconstituted type V collagen fibrils, where no reconstituted type V collagen fibrils have branches or merge with each other *in vitro* (*30*), either multiple contacts between the fibrils aligned in parallel or factors that promote the interlinking of type V collagen fibrils or both have to be taken into

consideration in order to explain the condensation of type V collagen fibrils. We assume that these fibrils are interlinked with each other by the factors that can bind to them.

Some extracellular matrix components such as tenascin C and PG-M (versican) show anti-adhesive property (*41–43*), in that the cells do not reach confluence, indicating that these materials prevent cell attachment to the culture dish. The molecular mechanism of this anti-adhesiveness is unknown. We speculate from our studies on type V collagen fibrils that the adherence of so-called anti-adhesive molecules to the cells could cause cell detachment from the dish. In order to exert such an influence on cells, these molecules may well bind with the cells. The following are the possible mechanisms by which such cell-binding substances exhibit anti-adhesive properties. By masking receptors for cell adhesion, anti-adhesive molecules may reduce the number of active receptors available for adhesion to culture dishes and/or may overwhelm the binding capacity of cells to culture dishes. Such mechanism(s) could be extended to some other anti-adhesive matrix molecules.

The present findings may have the following physiological relevance. As stated in the introduction, type V collagen in mature tissues appears to be co-localized with type I collagen presumably as hybrid fibrils (*5*, *18–20*). Thus, it is rather unlikely that homotypic type V collagen fibrils exist in mature tissues. However, in the developmental stages and during the initial stage of wound healing, the production and deposition of type V collagens precede those of type I collagen, implying that homotypic type V collagen fibrils exist in tissues in such cases (*44*, *45*). Further, our studies suggest that type V collagen is located on the surface of collagen fibrils (*18*, *24*). Thus, the type V collagen fibrils may well be in direct contact with the fibroblasts or mesenchymal cells *in vivo* under certain conditions. Type V collagen fibrils could well be involved in the tissue organization of various organs in which some condensation of fibroblasts or mesenchymal cells takes place before tissue organization is initiated.

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CONFLICT OF INTEREST

None declared.

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