The Reconstituted Type I Collagen Fibrils from Skins of Variously Aged Bovines Exert Different Effects on the Morphology and Integrin \(\alpha 2\beta 1\) Expression of Fibroblasts in Culture

Kaori SATO\(^1\), Tetsuya EBIHARA\(^1\), Eijiro ADACHI\(^2\), Seiichi KAWASHIMA\(^4\), Shunji HATTORI\(^{1,2}\) and Shinkichi IRIE\(^{1,2}\)

\(^1\)Nippi Research Institute of Biomatrix, and \(^2\)Department of Biomatrix Engineering, Japan Institute of Leather Research, 
\(^3\)Department of Molecular Morphology, Kitasato University Graduate School of Medicine, 
\(^4\)Department of Molecular Biology, Tokyo Metropolitan Institute of Medical Science

Received, 15 December 1998; accepted, 2 December 1999.

Abstract: We examined the effect of type I collagen extracted from the variously aged bovines when used as the substrate for skin fibroblast culture. We could not find any apparent difference in the cell adhesion and the expression of integrin \(\alpha 2\beta 1\) in the fibroblasts cultured on the non-fibrous form of type I collagens from the variously aged animals. But some prominent differences were observed between the collagens from young and old animals when the cells were cultured on the reconstituted type I collagen fibril. The morphology of reconstituted collagen fibrils was different depending on the animal age. The collagen isolated from young bovine skin assembled into thick fibrils, whereas the collagen from aged bovine assembled into thin fibrils. The fibroblasts cultured on type I collagen fibrils adhered on fibril surface from all aged animals. But they spread well only on the fibrils from young bovine collagen whereas the cell spreading was not observed on the fibril from 8-year bovine collagen within 1 h. Gel contraction was examined by the in-gel culture. No gel contraction occurred in the collagens from 18 month- and 8 year-old bovines, while the contraction of the gel occurred in the collagens from bovines younger than 3-month old. To investigate the molecular mechanism underlying these phenomena, the expression of the most responsible adhesion molecule, integrin \(\alpha 2\beta 1\), was examined by immunoblotting. The expression of integrin \(\alpha 2\beta 1\) in fibroblasts was higher in the collagen gels from young animals than that from aged animals. These results show that the collagens undergo different age-dependent molecular assembly and exert different effects on the features of cell adhesion.

Key words: collagen, aging, gel contraction, integrin \(\alpha 2\beta 1\), fibril

INTRODUCTION

Type I collagen is well known as the most abundant protein among the constitutive proteins of animal connective tissues. The number of cross-links between lysine residues in type I collagen\(^2\) and the lysyl oxidase activity in skin\(^2\) increase during growth and aging, strongly suggesting the accumulation of cross-links in the skin collagen of aged animals. We compared the extractability and fibril formation of the type I collagen from the variously aged animals, and showed the age-dependent increase in polymer type I collagen content, the accelerated rate of fibril formation, and the difference in the final absorbance (at 530 nm) of the collagen fibrils with the same rate of fibril deposition\(^3\). These results suggest that the function of collagens in various collagen-expressing tissues is changed by aging, and raise the possibility that the aging of animals affects the interaction of collagens with the cells.

It was also reported that both the fibroblasts from aged animals and the aged fibroblasts after many passages have less contractile activities in collagen gels than that from young animals and the cells in early passages, respectively\(^4\)\(^\text{-}^\text{6}\). However, whether the interaction of fibroblast with collagens depends on the crosslinking in collagens has not yet been clarified. Thus, we examined the effect of type I collagen from variously aged animal on the adhering behavior of fibroblasts. Among the integrins, the expression of integrin \(\alpha 2\beta 1\) increased during the collagen gel contraction\(^6\). In the inhibition assay of gel contraction by anti-integrin antibodies, an anti-\(\alpha 2\beta 1\) antibody inhibited the contraction while an anti-\(\alpha 1\beta 1\) antibody showed no effect\(^7\). Combining these reports, integrin \(\alpha 2\beta 1\) seems to be the most important cell adhe-
sion molecule functioning during gel contraction, and we determined the expression of integrin α2β1 during gel contraction of collagens derived from bovines of various ages.

MATERIALS AND METHODS

Materials

Proctase, an aspartic protease isolated from Aspergillus niger var. macrosporus was purchased from Meiji Seika, Ltd. (Tokyo, Japan). Polyclonal antibodies against the C-terminal peptides of human integrins α2 and β1, and an anti-actin monoclonal antibody were obtained from Chemicon International, Inc. (Temecula, CA, USA). Other chemicals were products of Wako Pure Chemical Industries, Inc. (Osaka, Japan).

Preparation of type I collagen from bovines of various ages

Bovine skins were obtained from several farms in the eastern areas of Hokkaido, Japan. Ages of bovines used were fetus, newborn, 3 month-, 18 month-, and 8 year-old. The hair and lipids were carefully removed from 100 g of bovine skin and washed overnight at 4°C with the washing buffer containing 10 mM disodium phosphate, pH 7.6, 1 mM N-ethylenediaminetetraacetic acid (EDTA), 0.1 mM N-ethylmaleimide, and 10 μM phenylmethylsulfonyl fluoride (PMSF). Washed skin was minced and homogenized in the fresh washing buffer, and centrifuged at 10,000 × g for 30 min at 4°C. Pellets were washed twice with the washing buffer, suspended in 10 volumes of diethyl ether-ethanol (mixed at 1:1) to remove the lipids, and filtered using a filter paper. This washing was repeated at least 3 times until the supernatant became colorless. The final pellets were air-dried and stored at −20°C. Collagen was extracted from the dried skin with the extraction solution containing 0.1 M tartaric acid −0.1 M sodium tartarate, pH 2.8, and 0.1 mg/mL proctase to obtain the proctase-solubilized collagen. Extraction was repeated 3 times for 1 day. The hair and lipids were carefully removed from 100 g of bovine skin, and the specimens were washed in the washing buffer and centrifuged at 10,000 rpm for 30 min at 4°C. To the supernatant were added equal volumes of 4 M NaCl in 50 mM acetic acid, and the mixtures were incubated overnight at 4°C and then centrifuged. The pellets were resuspended in 10 volumes of 50 mM acetic acid and incubated overnight. After removal of the insoluble materials by centrifugation at 10,000 rpm for 30 min at 4°C, a 1/5 volume of 5 M NaCl was added to the supernatants. After centrifugation, the pellets were resuspended in 10 volumes of 50 mM Tris-HCl, pH 7.5, containing 0.45 M NaCl, incubated overnight, and centrifuged. The supernatants (type I and Type III collagens) were dialyzed against 50 mM Tris-HCl, pH 7.5, containing 1.2 M NaCl, and centrifuged again. To the supernatants (type I collagen-rich fractions), HCl was added to adjust the pH to 3.5, and the insoluble materials were collected by centrifugation. The final type I collagen preparations were subjected to SDS-PAGE on 5% polyacrylamide gel containing 3.6 M urea to check the purity. The type I collagen fraction was dialyzed against 50 mM acetic acid, filtered through a 0.8 μm pore filter, and stored at 4°C. The protein contents were determined by BCA protein assay reagent (Pierce, Rockford, IL, USA) using 1 mg/mL type I collagen solution as a standard.

Electron microscopy

The type I collagens purified from fetal, 3-month and 8-year bovines were suspended at 1 mg/mL in the Dulbecco’s modified Eagle’s medium (DMEM; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 20 mM Hepes-NaOH, pH 7.2, adjusted by addition of NaHCO3 to pH 8.0, and were incubated for 1 h at 37°C to form the fibrils. The collagen fibrils were pre-fixed with 3.7% paraformaldehyde containing 0.02% picric acid for 30 min at 37°C to avoid dissolution of the fibril at low temperature, and fixed with 2.5% glutaraldehyde containing 0.2% tannic acid for 30 min. The fibrils were then stained with 0.5% uranyl acetate (pH 4.4) and phosphotungstic acid (pH 1.8) for the observation by transmission electron microscopy50. For the observation by scanning electron microscopy, the collagen gels were fixed with modified Zamboni’s solution containing 0.2% picric acid and 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 30 min. They were then cut with a razor blade to reveal the internal structure of gels. The specimens were fixed again for 30 min with 1% osmium tetroxide in Zamboni’s solution. They were dehydrated with ethanol and dried in a critical point dryer (Hitachi HCP-2, Tokyo, Japan). The dried specimens were placed on specimen stages using a piece of double-sided adhesive tape and coated with platinum in a sputter coater (Model 108 auto, Cressington Co., England). All the specimens were observed under a scanning electron microscope (Hitachi S-4500, Tokyo, Japan).

Adhesion assays

Neonatal human foreskin fibroblasts were maintained in DMEM supplemented with 10% fetal calf serum (FCS), 100 units/mL penicillin G sodium, and 100 μg/mL streptomycin. Fibroblasts were subcultured after treatment with 0.05% trypsin −0.2 mM EDTA at 37°C for 5 min, under which no degradation of integrin α2β1 occurred when examined by FACScan. To coat the culture plate with type I collagens as a non-fibrous form, collagens (10 μg/mL) were suspended in 5 mM acetic acid on 12-well suspension culture plate for 2 h at room temperature, and blocked with phosphate-buffered saline (PBS) containing 20 mg/mL bovine serum albumin (BSA). Fibroblasts were trypsinized, resuspended to 1 × 10^5/mL cells in DMEM containing 1 mg/mL BSA and 20 mM Hepes-NaOH, pH 7.2, placed on collagen-coated plates in duplicate, incubated for 10 min at 37°C, and washed three times with DMEM. Cells attached to each plate were counted using cell counting kit (Wako Pure Chemical, Osaka, Japan). It is based on the colorimetric method using WST-1 tetrazolium which is cleaved by mitochondrial enzymes in living cells and the product was detected by absorption at
a wavelength of 415 nm. Adhered cells were resuspended in 500 µL DMEM, and 25 µL of the reagent was added to this mixture followed by incubation for 1 h. The culture supernatants were transferred to 96-well in triplicate, and the absorbance was measured using a microtiter plate reader. The rate of the adhesion was calculated as follows: absorbance of wells with medium alone was subtracted from those with cell culture medium. 100% adhesion was arbitrarily defined as an average absorbance of non-washed wells.

On-gel culture on type 1 collagen

The type I collagens from fetus, newborn, 3-month, 18-month, and 8-year-old bovines suspended at 0.8 mg/mL in DMEM containing 1 mg/mL BSA, 20 mM Hepes-NaOH, pH 7.2, were adjusted to pH 8.0 by addition of NaHCO₃. The collagen solutions were left to form gels in 24-well plates by incubation at 37°C for 30 min. The 24-well plates were coated with 2% BSA dissolved in PBS as a control for 30 min at 37°C. A suspension of fibroblasts (10⁴ cells) in DMEM supplemented with 1 mg/mL BSA were overlaid. After incubation for another 23 h. Supernatants were transferred to 96-well in triplicate reads from those with cell culture medium. The morphology of reconstituted fibrils of collagens derived from aged and young bovines

The proctase-solubilized collagen α chains are free of telopeptides (Sato, unpublished result) and consists of essentially only triple-helical region. Using this solubilized collagen, the role of triple helical region on fibril formation and gel contraction was examined. We reported that the absorbances at 530 nm of the reconstituted collagen fibrils were different from each other. Depending on the age of the animal source, suggesting the morphology of the fibrils are different. First we examined the shapes of reconstituted the proctase-solubilized collagen fibril using electron microscopy (Fig. 1, transmission electron microscopy). The type I collagens isolated from the skins of fetal, 3-month, and 8-year-old bovines were allowed to form the fibrils in vitro. The diameter of the collagen fibril from fetal bovine was very thick (150–300 nm) compared to the 3-month (80–100 nm), and 8-year old collagen fibrils (40–70 nm) (Fig. 1). All of the fibrils (collagens from fetal, 3-month, and 8-year old bovine) showed a collagen-specific 67 nm-period arrangement, implying that the collagen molecules assemble with each other in the three-dimensional ordered structure. Super fibril structures of collagens were also examined by scanning electron microscopy (Fig. 2). The fibrils from fetus showed a straight and mesh-like (Fig. 2, A and D) structure, while the fibrils from 3-month and 8-year old bovine had a twisted and sponge-like structure (Fig. 2, B, C, E, and F).

Adhesion of human skin fibroblasts to type 1 collagen from variously aged bovine in the non-fibrous forms

Next, we examined the effect of collagens in the non-fibrous forms on the fibroblasts. Human neonatal skin fibroblasts were cultured on the dish coated with collagens from the different aged animals in the non-fibrous form. Half of fibroblasts adhered on collagen-coated surface of all preparations within 10 min, but there was no adhesion on bovine serum albumin (BSA) coated surface (Fig. 3). The degree of adhesion on collagens after 10 min varied between 54.8% and 62.5%, but there was no significant difference among the collagen preparations. After 30 min almost all fibroblasts adhered onto any collagen preparations (data not shown). This means that the fibroblasts do not discriminate collagens from bovines of various ages as far as in
Fig. 1. Fibril thickness of type I collagens purified from fetal-, 3-month, and 8-year old bovines.

Type I collagens purified from fetal- (A–C), 3-month (D–F), and 8-year (G–I) old bovines by proctase treatment were allowed to form fibril and observed by transmission electron microscopy. Fibrils from fetus were very thick (A), those from 3-month old bovine were intermediate (D), and those from 8-year bovine were thin (G). The fibrils in A, D, and G are magnified in B, E, and H, respectively. The 67 nm staggered arrangement of collagen molecules is indicated by arrowheads. The vertical sections of fibrils shown in C, F, and I, indicate that almost all fibrils are those of the column type. Bar, 200 nm.
Reconstituted fibrils from type 1 collagens isolated from fetus (A, D), 3-month (B, E), and 8-year (C, F) old bovines were observed by scanning electron microscopy. The fibrils of fetal collagen (A) was straight, and the those of 3-month (B) and 8-year (C) old bovines were twisted each other. Bar, 2 μm. The fibrils were observed in high magnification (D–F). Several fibrils of collagens from 3-month and 8-year old bovines assembled each other to thick fibrils (E and F). The single fibrils are indicated by arrowheads. Bar, 400 nm.

the form of non-fibrous molecule.

The fibroblasts spread well on every collagen preparations and showed very similar morphology among them, but did not spread on BSA-coated plate even after 16 h (Fig. 4A). The expression of integrin α2 in fibroblasts cultured for 16 h on the collagens in a non-fibrous form was examined by immunoblot analysis (Fig. 4B). The amount of expressed α2 was not different among fibroblasts cultured on the collagens from bovines of different ages, compared to the amount of actin as a control.

**Cell spreading on reconstituted collagen gels**

It was observed that the morphology of reconstituted collagen fibrils was different among collagens from bovines of various ages (Figs. 1 and 2). Therefore, we examined the effects of reconstituted collagens on the fibroblast adhesion to and spreading on collagen gels at 1 h culture in serum-free medium (Fig. 5). Fibroblasts attached to all collagen gel surface same as a non-fibrous form regardless of animal ages, but their spreading and the network formation between the cells were obviously different from each other. The microspike structures protruding from the fibroblasts were long when cultured in the collagens from young bovines, but they were short when cells were cultured in collagen from old bovines, especially 8-year old bovine.

**Three dimensional collagen gel contraction**

Next, gel contraction induced by cultured fibroblasts was examined using the type I collagens isolated from the skins of fetal, newborn, 3-month, 18-month, and 8-year
old bovines (Fig. 6). Upon the in-gel culture with proctase-solubilized collagen for 24 h, fibroblasts contracted the collagen gels isolated from the young bovines (fetal, newborn, and 3-month old), but not the collagen gels from the older animals (18-month and 8-year old).

The morphology of fibroblasts cultured for 24 h in the collagen gel is shown in Fig. 7. It is a prerequisite for gel contraction by fibroblasts to form a cell-cell adhesion-like mesh with cell spikes (meshwork). Upon the in-gel culture with the collagens from fetal, newborn, and 3-month old bovines, the cells formed meshwork. However, the cell-cell adhesion was weak in those collagens from the 18-month and 8-year old bovines, in which fibroblasts could not induce gel contraction as described above, and the morphology of cells was different from that of cells in the collagens from young animals.

**Amount of integrins in gel culture**

It was reported that interaction of the fibroblast with collagen is regulated through the collagen receptor, integrin α2β1 (b). We estimated the amount of integrins α2 and β1 by western blot analysis (Fig. 8). A large amount of integrin α2 was detected in fibroblasts cultured in the collagen gels from young animals, whereas only a small amount in fibroblasts cultured in collagen gels from older animals after 48-h culture (Fig. 8A). The expressions of integrin β1 were similar to α2, and a large amount of integrin β1 was detected in fibroblasts cultured in the collagen gels from young animals, whereas less amount of integrin β1 was detectable in fibroblasts cultured in the collagen gels from older animals after 48-h culture (Fig. 8B). The amounts of actin as a control remained unchanged during the culture in any of the collagen gels. The induced amounts of α2 in fibroblasts cultured were the highest in gels, and next on gels. By densitometrical analysis, the detected amounts of integrin α2 in fibroblasts cultured in DMEM supplemented with 10% FCS for 24 h in the collagen gels were 9.9-fold from

---

**Fig. 3. Adhesion of fibroblasts on non-fibrous type I collagens purified from fetal, newborn, 3-month, 18-month, and 8-year old bovines.**

A 12-well suspension culture plate was coated with type I collagens (10 μg/mL) purified from fetus (Fe), newborn (NB), 3-month (3 m), 18-month (18 m), 8-year (8 y) old bovines, or control (bovine serum albumin, BSA) for 2 h at room temperature, and blocked with 20 mg/mL BSA. Fibroblasts were trypsinized and resuspended at 1×10⁴ cells/mL in DMEM containing 1 mg/mL BSA, and plated on the well. Fibroblasts were incubated at 37°C for 10 min, and non-adhered cells were washed out three times with DMEM. Cells attached to each plate were counted using cell counting kit. The adhesion was arbitrarily defined as an average absorbance of non-washed wells as 100%.

**Fig. 4. Morphology and integrin α2 expression of skin fibroblasts cultured on non-fibrous collagens.**

A 35 mm culture plate was coated with type I collagens (10 μg/mL) as in Fig. 3, and blocked with 20 mg/mL BSA. Fibroblasts were trypsinized and resuspended in DMEM containing 1 mg/mL BSA and 20 mM Hepes-NaOH, pH 7.2. Fibroblasts (10⁴ cells) were cultured for 16 h and photographed (A). Bar, 100 μm. The cells were lysed with RIPA buffer containing 1% Triton X-100. Soluble lysates were subjected to SDS-PAGE and immunoblotting using an anti-α2 integrin antibody (B). Fibroblasts were cultured on control BSA (lane 1), on collagens purified from fetus (lane 2), newborn (lane 3), 3-month (lane 4), 18-month (lane 5), or 8-year (lane 6) old bovines.
Fig. 5. Spreading of fibroblasts on collagen gels.
Proctase-solubilized type I collagens from variously aged bovines (1 mg/mL) were suspended in DMEM containing 20 mM Hepes-NaOH, pH 7.2, and 1 mg/mL BSA, and incubated at 37°C for 30 min to complete gel formation. Fibroblasts were then cultured on collagen gels purified from fetus, newborn, 3-month, 18-month, or 8-year old bovine, and photographed after a 1-h culture. Bar, 50 μm.

fetal bovine, 8. 8-fold from 3-month, 8. 6-fold from 8-year bovine, respectively, of non-fibrous collagen control, and that of integrin α2 in fibroblasts cultured on the collagen gels were 7. 4-fold from fetal bovine, 7. 2-fold from 3-month, 6. 3-fold from 8-year bovine, respectively, of non-fibrous collagen control (data not shown). While the difference between in-gel or on-gel culture and on non-fibrous collagen was clear, the difference among animal ages was moderate.

**Through-gel migration of fibroblasts**

When fibroblasts were cultured in collagen gel from 8-year old bovine, cells were moved to the bottom during the culture (data not shown). To examine this phenomenon more precisely, we performed the following experiment. Type I collagens from the bovines of various ages were poured onto cover-glasses and allowed to form gels as thin films. Fibroblasts were cultured on the gels in a serum-free medium. After 48 h, the gels were mechanically detached from the cover-glasses, and cells on the gel surfaces and those on the cover-glasses were observed under a microscope. As shown in Fig. 9, almost all cells remained attached on the gel surface of collagen from the young bovines (fetus and newborn) and formed meshwork, while many cells were detached from the collagen gels derived from animals older than 3-month old and found on the glass surface. Since fibroblasts were found to attach once within 1 h of the culture on any collagen gels (Fig. 5), the cells found at the surface of cover-glass are likely to be those that had moved through the collagen gels during 2-day culture.

**DISCUSSION**

Our previous study demonstrated the increase of crosslinking within collagen triple helical region on animal aging[3]. When the electrophoretic analysis after heat-denaturation of the proctase-solubilized type I collagens from fetal, newborn, 3-month, 18-month, and
Effect of Animal Aging on Type I Collagen

Fig. 7. Morphological change of skin fibroblasts cultured in collagen gels from young and aged bovines.
Fibroblasts at 4×10⁴ cells/mL were cultured in DMEM medium containing 10% FCS and 0.8 mg/mL proctase-solubilized type I collagen gels purified from fetus, newborn, 3-month, 18-month, or 8-year old bovines. Phase-contrast micrographs were obtained after a 24-h culture. Bar, 100 μm.

Fig. 6. Three-dimensional gel contraction of proctase-solubilized type I collagens by skin fibroblasts.
Fibroblasts were trypsinized and resuspended at 4×10⁴ cells/mL in DMEM medium containing 10% FCS and 0.8 mg/mL proctase-solubilized type I collagen, and cultured in 24-well plate (15 mm diameter) precoated with 2% BSA. Collagen gel diameters were measured using a ruler after 24-h and 48-h cultures.

8-year old bovines was done, α chain content decreased depending on the aging, while high molecular weight forms which were consisted of β and γ chains increased in collagen of aged animals⁹. These collagen prepared from variously aged bovines showed different characteristics in fibril formation in vitro. Compared to the collagens from young animals, those from the aged one required a shorter time to complete fibril formation and showed lower final turbidity⁹. The diameter of type I collagen fibrils from aged animals were thinner than that

Fig. 8. Amount of integrin α2 and β1 in fibroblasts cultured in the gel of type I collagens from young and aged bovines.
Fibroblasts (4×10⁴ cells/mL) suspended in DMEM containing 10% FCS and 0.8 mg/mL proctase-solubilized type I collagen were plated on 24-well plate (16 mm diameter) precoated with 2% BSA, and cultured for 48 h. Used type I collagen was purified from fetus (lane 1), newborn (lane 2), 3-month (lane 3), 18-month (lane 4), or 8-year (lane 5) old bovines. After cells in collagen gels were removed from wells, both of the cells in gels and adhered on dishes were washed twice with PBS, lysed in RIP A buffer containing 1% Triton X-100, 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 150 mM NaCl, and 0.5 mM phenylmethylsulfonyl fluoride for 10 min at room temperature, and centrifuged at 15,000 rpm for 5 min at 37°C. The supernatant was boiled and subjected to SDS-PAGE on 7.5% polyacrylamide gel. After the electrophoresis, the proteins were transferred onto a PVDF membrane, and immunostained with anti-α2 integrin (A), anti-β1 integrin (B), or anti-actin (C) antibodies.
from fetus collagen, and their morphology was different from each other (Figs. 1 and 2). The lower turbidity may be due to the thinner fibril. The reason why the collagen from aged animal assembled into thinner fibril is not clear yet. Post-translational modification such as glycosylation on hydroxylysine residues is one of candidates to determine the fibril thickness9, but the content of hydroxylysine residues, which was thought to parallel the extent of glycosylation of collagen10, was not changed among type I collagens isolated from fetal to 8-year old bovine skins (data not shown).

The skin fibroblasts could adhere and spread well on non-fibrous type I collagens regardless the originated animal ages. No alteration in the protein level of integrin $\alpha_2\beta_1$ was also observed in fibroblasts on non-fibrous collagens from bovines of all ages examined (Fig. 4B). These results mean that cells cannot distinguish the collagens in non-fibrous states from young and aged animals. However, the collagen-dependent cell behavior was different when the cells were cultured on-collagen gel or in-collagen gel. Upon on-collagen-gel culture, fibroblasts adhered onto all collagen gels from bovines of any ages within 1 h, however, the cells could not spread on the gels from 8-year old bovine collagen while cells spread and formed cell-cell contact spikes on the collagen from bovines younger than 18-month (Fig. 5). These results suggest that the out-side-in signal from non-fibrous collagen is different from that from 3-dimensional collagen fibril.

Upon in-collagen-gel culture, the difference was more prominent. Contact of fibroblasts to collagens from old bovines (18-month and 8-year) seemed weaker than that of cells to collagen from young bovines (fetus and newborn). In the collagen gels from young animals, fibroblasts had dendritic shape and extended cell spikes into the gel to form the meshwork structure between the cells (Fig. 7, fetus and newborn). On the other hand, fibroblasts did not spread and aggregate in the collagen gels from old animals (Fig. 7, 18-month and 8-year). When we examined the integrin $\alpha_2\beta_1$ in the fibroblasts cultured in collagen gel, not like the case on the non-fibrous collagen, the amount of integrin $\alpha_2$ was different depending on the animal ages. Integrin $\alpha_2$ was abundant in the fibroblasts cultured in the collagens from young animals (Fig. 8). Since integrin $\alpha_2$ is expected to combine with $\beta_1$ and the amount of resulting integrin $\alpha_2\beta_1$ determines the affinity of cells to collagen, the fibroblasts may have higher affinity to the collagen from young
animals than that from aged animals. In addition, fibroblasts cultured on the gels of collagens from young animals formed cell meshwork within 1 h and remained attached to the gel surface even after 2 days. On the other hand, only a few cells formed the meshwork structure on-and in-collagen-gels of old animals within 1 h, and most of the cells failed to remain on gels and migrated or fell through the gels, then were found attached to the glass-surface at the bottom after 2 days (Fig. 9). Then we compare the behaviors of the cells on non-fibrous collagen and in collagen fibril. On non-fibrous collagen, cell adhesion and the amount of integrin α2β1 did not change among collagens from animals of any ages. This may suggest that the amount of integrin α2β1 is not an essential factor for determining the cell adhesion force to the coated collagen and also suggest that a relatively small number of integrin molecules participate at the place where cells adhere to the substrate collagen. On the other hand, the cell may need a larger number of integrin molecules for the gel contraction and regulation of cell shape, because that the amount of integrin α2β1 and gel contraction were different in the collagen fibrils depending on the age of bovine. The fibrils consisted of the collagens from young animals may have a higher ability to induce the collagen-specific integrin than that from old animal. Since the fibril structure of collagen from old animal is thin, the binding site in the collagen receptor of cells may be hidden or the thin collagen fibril is not strong enough to support the cell binding, and thus the induction of integrin α2β1 is inhibited. Yamato and Hayashi reported that the topological distribution of α2β1 integrin on fibroblast cultured in collagen gel was different from the focal adhesion plaque formed on the coated collagen. In collagen gel, integrin β1 and vinculin localized over the entire surface of the cells dispersively. The difference of the fibril shape may affect the distribution and the quantity of the collagen receptor on the cell. The relation between the cell shape and the integrin α2β1 expression was also reported using the α2β1 integrin gene expression in three-dimensional collagen lattices and mediates the reorganization of collagen fibrils. J. Cell Biol., 115, 1427–1436


Acknowledgment: We thank Dr. Takemaru Abe, Mitsubishi Chemical Corporation (Nakashibetsu, Hokkaido, Japan) for providing the bovine skins.

References


