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Gelation of the Lens Capsule Type IV Collagen Solution at a Neutral pH[1]

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It is known that the type IV collagen extracted from EHS tumor assembles under a physiological condition, but not in a gel form. The EHS type IV collagen requires the other basement membrane components, laminin1, heparansulfate proteoglycan, and/or nidogen for gelation. On the other hand, Muraoka *et al.* reported that the bovine lens capsule type IV collagen alone gelated under a unique and unexpected condition of 2 M guanidine-HCl and 50 mM dithiothreitol, a condition which is thought to be dissociative for most biological macromolecules, including extracellular matrix [Muraoka, M. *et al.* (1996) *J. Biochem.* 119, 167-172]. The present report shows that the bovine lens capsule type IV collagen formed a gel under physiological conditions of pH and ionic environment, though the apparent rigidity of the gel was weaker than that of the gel formed in 2 M guanidine-HCl and dithiothreitol. The rigidity depended greatly on the incubation temperature and NaCl concentration of the type IV collagen solution, as observed in terms of the contractility of gel volume under centrifugal force. The gel formed in 150 mM NaCl and 20 mM phosphate, pH 7.3, at 28°C contracted to 20% of the original volume on centrifugation of 1,800×*g* for 10 min, while the gel formed at 4°C, where type I collagen did not gelate at all, retained 90% of the original volume at the same centrifugal force. NaCl concentration was another important factor influencing the mechanical properties of type IV collagen gel. The gel formed at 150 mM showed maximal rigidity in the range of 0 to 300 mM in terms of the contractility on centrifugation. An image of a Pt/C replica of the gelated type IV collagen reconstituted at 4 or 28°C in 20 mM phosphate, pH 7.3, containing 150 mM NaCl showed fine meshworks consisting of rather homogeneous pore sizes, resembling the skeletal structure of basal lamina. Since the condition where the type IV collagen alone formed gels was physiological in terms of ionic strength and pH, the aggregate structure and gel properties might reflect the *in vivo* type IV collagen supramolecular structure and the property.

Key words: basement membrane, gel, lens capsule, self-assembly, type IV collagen.

Lens capsule comprises a basement membrane of lens epithelial cells. A portion of the type IV collagen, a major component of the basement membrane, can be extracted nonenzymatically with 0.5 M acetic acid from lens capsules (*1-4*). Several reports on EHS tumor type IV collagen have indicated that the extracted molecules reassemble under physiological conditions, but not into a gel form in the absence of the other basement membrane components (*5, 6*). Most recently, Muraoka *et al.* reported that purified type IV collagen from bovine lens capsule, without other basement membrane components, assembled into a gel form in 2 M guanidine-HCl and 50 mM dithiothreitol (*7*). The study demonstrated for the first time that the type IV collagen alone could assemble to a gel form. In general, guanidine-HCl is a potent dissociation reagent for most biological macromolecules. In fact, Kleinman *et al.* used guanidine-HCl and dithiothreitol for extraction of the type IV collagen from EHS tumor tissue (*8*). Thus, the aggregation of the bovine lens capsule type IV collagen in guanidine-HCl and dithiothreitol may imply that the lens capsule type IV collagen molecule possesses a unique interacting property, distinct from that of the EHS tumor type IV collagen. In spite of the new finding on the gelation of type IV collagen, the assembled structure in guanidine-HCl and dithiothreitol can not be directly related to that of type IV collagen *in vivo*. For comparing basement membrane scaffolding *in vivo* with the type IV collagen aggregate structure *in vitro*, it would appear to be prerequisite that self-assembly of type IV collagen proceeds under physiological or physiologically possible conditions. Veis *et al.* (*1-3*) examined self-assembly of bovine lens capsule acid extract, which contained the type IV collagen as a major component, under physiological conditions (100 mM phosphate). They showed that a neutral pH solution of the type IV collagen at 4°C gradually became turbid upon

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Abbreviations: EHS, Engelbreth-Holm-Swarm; NC, non-collagenous; SDS, sodium dodecylsulfate.

warming at 28°C for 30 min, though turbidity did not appear if the temperature was kept at 4°C. They considered that the increase in turbidity on incubation at 28°C was due to the assembly of the isolated molecules (*3*). Kleinman *et al.* reported that the whole extract of EHS tumor tissue, containing laminin as a major component, nidogen, proteoglycan and the type IV collagen, formed a gel under physiological ionic strength and pH at 36°C, but that the type IV collagen alone did not (*6, 9*).

Here we show that the type IV collagen obtained from bovine lens capsule aggregated in the form of a gel on incubation at physiological ionic strength and pH. Rigidity of the gels depended on the gelation conditions, particularly temperature and NaCl concentration. The fine structure of the gel was examined with a transmission electron microscope in comparison with the skeletal structure of basement membranes *in vivo*.

MATERIALS AND METHODS

Materials—The lens capsules isolated from bovine eyes were kindly supplied by Nitta Gelatin, Osaka. Trizma base, pepstatin A and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical. Urea (specially prepared reagent) and *N*-ethylmaleimide were purchased from Nacalai Tesque. Sodium azide, sodium chloride, sodium phosphates (monobasic, dibasic), and acetic acid were purchased from Wako Pure Chemical Industries. An extract with dilute HCl from rat tail tendon was used as type I collagen.

Methods—Acid extraction of type IV collagen from bovine lens capsule: Lens capsules were homogenized with a Polytron homogenizer in 0.5 M acetic acid with protease inhibitors consisting of 5 mM EDTA, 1 mM *N*-ethylmaleimide, 0.1 mM phenylmethylsulfonyl fluoride, and 1 μM pepstatin A, with 0.02% (w/v) sodium azide. The homogenate suspension was stirred for 2-3 days (5-10 ml per 1 g wet weight of lens capsules). The type IV collagen in the supernatant after centrifugation was precipitated by addition of NaCl to 1.2 M in the acidic solution (*10*). The precipitate was dissolved in 0.5 M acetic acid and the solution was dialyzed against 1 mM HCl and stored at 4°C until use. Protein concentrations were determined as moles of total amino acid residues obtained by amino acid analysis.

Measurements of relative rigidity of the gel in terms of volume change after centrifugation: The type IV collagen solution in dilute HCl was mixed with 4 M NaCl and 200 mM phosphate buffer, pH 7.3, in a glass tube (NICHIDENRICA GLASS, Kyoto) to give predetermined concentrations. After the incubation, the aggregates were in a gel form prior to centrifugation in all the conditions examined. Thus, mechanical fragility was arbitrarily measured in terms of the relative reduction in volume after centrifugation in a swinging rotor (TMS-4, TOMY SEIKO, Tokyo). The distance between the surface of the solution and the top of the gel was used for estimation of the reduction in gel volume, assuming that the tube consisted of a cylinder with a hemispherical bottom.

SDS-polyacrylamide gel electrophoresis: Polyacrylamide gel electrophoresis in SDS was performed essentially as described by Laemmli (*11*).

Quick-freeze, deep-etch replication, and electron microscopy: Samples of type IV collagen gels were analyzed by electron microscopy using the quick-freeze, deep-etch replication technique. The samples were placed on the plunger of the freezing apparatus and slammed onto a copper block precooled with liquid helium. The frozen gel was transferred to liquid nitrogen and then attached to the rotating specimen stage precooled to below −170°C in a BAF 400D freeze-etch apparatus (Balzers). They were then fractured, etched, and rotary shadowed with Pt/C at an angle of 20 degrees and coated with carbon at an angle of 85 degrees. The specimens were floated onto commercial bleach to digest the gel and rinsed in pure distilled water several times. The replicas were picked up on copper grids and air-dried. They were examined with an electron microscope, Hitachi H-7000 (Hitachi, Tokyo) operating at 100 kV. All the photographs were taken at 50,000 magnification.

RESULTS

SDS Polyacrylamide Gel Electrophoresis Analysis of the Proteins of the Bovine Lens Capsule Acid Extract—Bovine lens capsule acid extract, when subjected to electrophoresis on 5% SDS polyacrylamide gel without reduction, showed protein bands at the top of the running gel with Coomassie Brilliant Blue R-250, suggesting the existence of high-molecular-weight polypeptides. When the sample was reduced, three distinct bands were seen with molecular weights of 180K, 175K, and 160K in addition to a band with a molecular weight of 320K which may represent a β component (Fig. 1). All the bands except the dye front were immunostainable with a polyclonal antibody to the type IV collagen (*4, 7*). The amino acid analysis of the acid extract corresponded to that of type IV collagen (data not shown). The major protein in the acid extract was the type IV collagen, as reported previously (*3*).

Effects of NaCl Concentration, Temperature, Protein Concentration, and Incubation Time on the Contractility of the Type IV Collagen Gel—The type IV collagen from bovine lens capsule was incubated in 20 mM phosphate

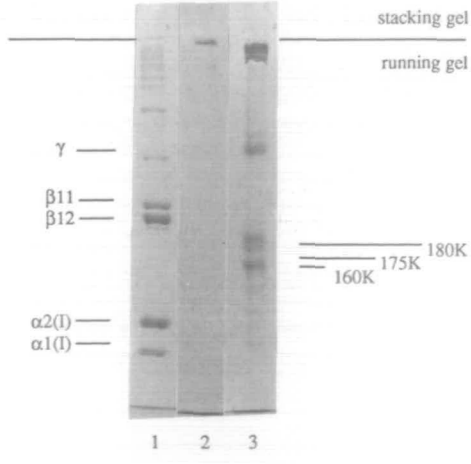

Fig. 1. **Characterization of the lens capsule acid extract by SDS-polyacrylamide gel electrophoresis.** An acid extract of bovine lens capsules was subjected to SDS-PAGE with (lane 3) or without (lane 2) reduction. Type I collagen from rat tail tendon (lane 1) was used as a molecular weight standard.

buffer, pH 7.3, with 0, 50, 150, or 300 mM NaCl at 4 or 28°C for 24 h. Relative volumes after centrifugation were obtained as shown in Fig. 2a. The reduction in volume of the type IV collagen gel at 4°C depended on the NaCl concentration in which the gel was formed. The gels formed with 0 or 50 mM NaCl shrank on centrifugation at 1,800×*g*. However, the gel formed with 150 or 300 mM NaCl retained more than 80% of the initial volume after the same treatment. On centrifugation at 4,250×*g*, the relative volume of the gel formed at 300 mM NaCl was smaller than that of the gel formed at 150 mM NaCl, suggesting that 150 mM NaCl was optimal for apparent rigidity of the reconstituted gel. The incubation temperature had a marked influence on the gel rigidity. The solution incubated at 28°C, regardless of NaCl concentration, formed only fragile gels which collapsed on centrifugation of 1,800×*g*.

To examine the effect of incubation temperature in more detail, type IV collagen solutions were incubated in 20 mM phosphate (pH 7.3) with 150 mM NaCl at 4, 15, 28, or 36°C for 24 h. As the incubation temperature was increased, relative volumes after centrifugation became smaller, indicating that the lower the incubation temperature was, the more rigid was the gel formed (Fig. 2b).

The effect of protein concentration on the gel properties was examined in the range of 0.49 to 0.99 mg/ml. At 0.99 mg/ml, the gel retained the original volume under 1,800×*g*. The volume became less than 25% at the same centrifugal force, when the protein concentration was below 0.70 mg/ml (Fig. 2c).

In 20 mM phosphate, pH 7.3, containing 150 mM NaCl, relative volumes after centrifugation were measured as a function of incubation time at 4 or 28°C. At 28°C, regardless of the time of incubation, the gel volume reduced to less than 20% of the original at a weak centrifugation force, 1,800×*g*. At 4°C, the relative volume of the gel formed by incubation for 8 h was about 20% at a centrifugal force of 1,800×*g*. However, after incubation for 20 h, the gel compressibility decreased and the gel volume was about 90% of the original under the same force (Fig. 2d). Under the conditions examined, the protein concentrations in the supernatants after centrifugation were roughly the same, based on the protein staining on SDS-PAGE (data not shown).

Effect of Incubation Temperature on Mechanical Properties of the Gel—Since apparent difference in macroscopical and mechanical properties of lens capsule type IV

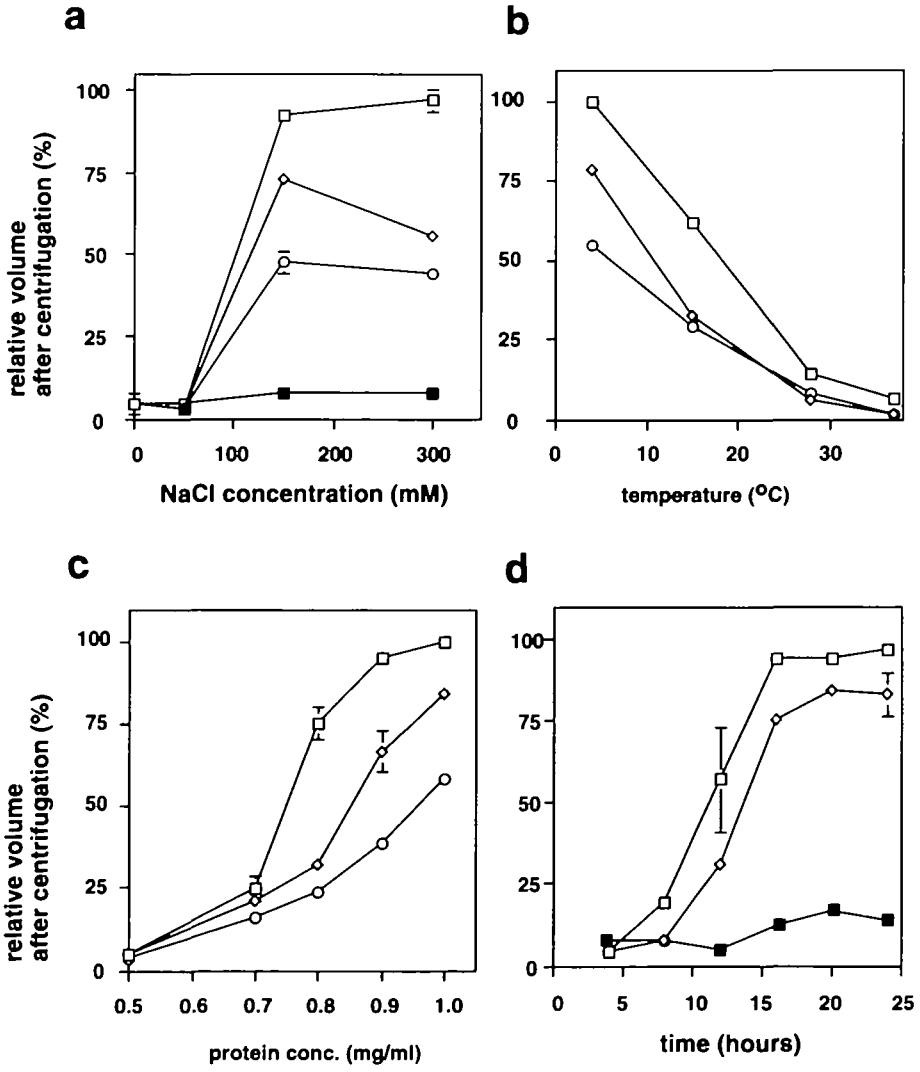

Fig. 2. **Volume changes with centrifugal force as a measure of the gel property.** The type IV collagen solution was incubated under the conditions indicated below, then the relative volume after centrifugation (□, ■, 1,800×*g*; ◇, 4,250×*g*; ○, 6,500×*g* for 10 min) was measured as described in "MATERIALS AND METHODS". (a) Effect of ionic strength. The lens capsule type IV collagen solution in acidic pH was mixed with 4 M NaCl and 200 mM phosphate, pH 7.3, in a glass tube to give a final phosphate concentration of 20 mM, a final protein concentration of 0.84 mg/ml and a final NaCl concentration of 0, 50, 150, or 300 mM. The relative volume of the gels was measured after centrifugation of the gels incubated for 24 h at 4°C (□, ◇, ○) or 28°C (■). (b) Effect of temperature. Solutions of type IV collagen were made to give final concentrations of 20 mM phosphate, pH 7.3, 150 mM NaCl and 0.99 mg of the protein/ml, followed by incubation at 4, 15, 28, or 36°C for 24 h. (c) Effect of protein concentration. The type IV collagen solutions (0.99–0.49 mg/ml) were incubated in 20 mM phosphate, pH 7.3, and 150 mM NaCl at 4°C for 24 h. (d) Time course of the change in gel property. Solutions of lens capsule type IV collagen were incubated in 20 mM phosphate, pH 7.3, 150 mM NaCl, 0.99 mg of the protein/ml at 4°C (□, ◇), or 28°C (■) for 4–24 h. Each point is the mean of two independent experiments.

collagen gel depended most markedly on the gelation temperature (Fig. 2, a, b, and d), we examined whether the effect of temperature was reversible or not by changing the temperature from 4 to 28°C and *vice versa*. The acid extract was incubated for 24 h at 4°C in 20 mM phosphate, pH 7.3, containing 150 mM NaCl, then the incubation temperature was changed to 28°C and incubation was continued for another 24 h. When the incubation temperature was changed from 4 to 28°C, the relative volume became 28% at $4,250 \times g$. On the other hand, the relative volume of the gel incubated throughout at 4°C was 78% at the same centrifugal force. The relative volume of the gel kept at 28°C was less than 20% at $1,800 \times g$, while the relative volume of the gel after centrifugation was increased by a temperature change from 28 to 4°C (Fig. 3). The rigidity as evaluated by volume compressibility under centrifugation depended on the temperature at the end of incubation as well as on the temperature during gelation.

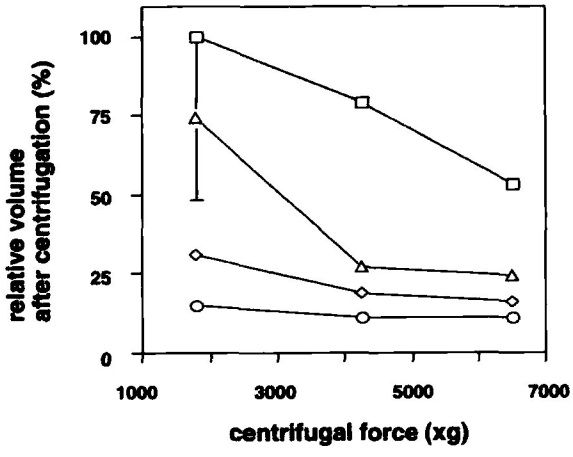

Fig. 3. **Effect of temperature on the gel rigidity.** The type IV collagen solution was mixed at 4°C with 200 mM phosphate, pH 7.3, 4 M NaCl to give final concentrations of 20 mM phosphate, 150 mM NaCl and 0.99 mg/ml protein, followed by incubation at 4 or 28°C for 24 h. Then, the temperature of incubation was changed and incubation was continued for another 24 h. The relative volumes after centrifugation were measured (□, 4→4°C; △, 4→28°C; ◇, 28→4°C; ○, 28→28°C). Each point is the mean of two independent experiments.

Supramolecular Structure of Collagen Gels—The change in gel volume was smallest when the gel was made in 20 mM phosphate buffer, pH 7.3, and 150 mM NaCl at 4°C. The gel made at 28°C collapsed under a weak centrifugal force. The supramolecular structure of the gel showed extensive meshwork under an electron microscope (Fig. 4). Branching and anastomosing of filaments were seen in the gels formed at 4°C and also at 28°C. These meshworks seemed to be formed by lateral association of type IV collagen, a thread-like structure approximately 400 nm long with globular structure designated as the noncollagenous globular domain (NC1) at the C-terminal. The mean lengths between branching points of meshworks formed at 4 and 28°C were 15.5 nm (s.d. 3.6 nm) and 14.5 nm (s.d. 3.0 nm), respectively. No statistically significant difference was seen between the meshworks formed at 4 and 28°C, with respect to interbranching spacings.

DISCUSSION

Muraoka *et al.* reported that the bovine lens capsule type IV collagen alone assembled to a gel form under conditions

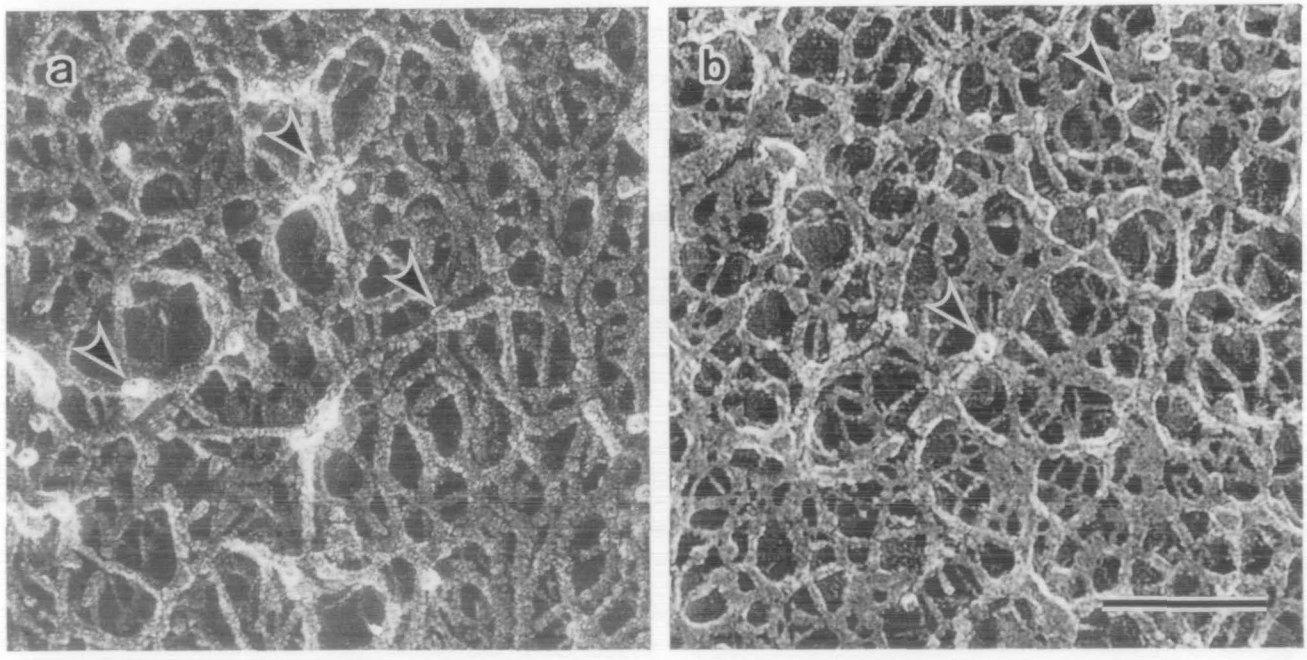

Fig. 4. **Quick-freeze, deep-etch replica image of type IV collagen gels formed at 4°C (a) and 28°C (b).** A solution of type IV collagen was incubated in 20 mM phosphate buffer, pH 7.3, containing 150 mM NaCl at 4°C or at 28°C for more than 24 h. The gels formed were quick-frozen, deep-etched and rotary shadowed with Pt/C. The replicas show meshworks with extensive branching and anastomosing as shown in a and b. The distances between branching points are 15.5 nm (s.d. 3.6 nm) in a and 14.5 nm (s.d. 3.0 nm) in b. Globular structures (arrowheads) are seen on the filaments in a and b. The magnification is ×210,000. Bar = 100 nm.

where the mouse EHS tumor type IV collagen was reported to be solubilized (*7, 8*). Since 2 M guanidine-HCl and 50 mM dithiothreitol in which the bovine lens capsule type IV collagen gelated, is far from the physiological condition, the structure of the type IV collagen aggregate as well as the intermolecular interactions may not reflect the *in vivo* situation. Here, we report that the type IV collagen preparation extracted from bovine lens capsule assembled in a gel form under physiological conditions. The acid extract of bovine lens capsule contained small amounts of two polypeptides, around 25K, which did not react with anti-type IV collagen antibody (*7*). The type IV collagen preparation free of the 25K polypeptides obtained by DEAE-Sepharose chromatography, gelated under the same conditions of pH and ionic strength even more readily (data not shown), indicating that type IV collagen assembly was the cause of the gelation.

Temperature-dependent behavior of the collagenous proteins has been studied for type I collagen, which comprises 95% of the triple helical (TH) domain. In neutral pH and 150 mM NaCl, the type I collagen stays in solution at 4°C. Upon incubation of the solution at 25°C or above, the proteins aggregate to form fibrils with a banding pattern owing to the D-staggered lateral association through the TH domains of the molecules. One interpretation of this phenomenon is that at a low temperature the molecules are dispersed due to hydrophilic interaction with the solvent or high hydration, while the dehydration caused by elevating the temperature is accompanied with lateral association of the TH domains. It is worth discussing whether or not the finding that the temperature-dependent gel rigidity (Figs. 2 and 3) could be accounted for by the TH domain behavior. As shown for the type I collagen, at a high temperature, *e.g.* 28°C, lateral association of the TH domain of the type IV collagen is expected to be enhanced along with dehydration, while at a low temperature, *e.g.* 4°C, lateral association may be repressed owing to strong hydration.

Irreversible contraction of the gel on centrifugation would imply a heterogeneous distribution of the laterally associated collagen molecules. That is, the proteins are densely packed in some regions of the gel matrix and in other regions the proteins are sparse. The dense regions would tend to sediment under the influence of centrifugal force. Since the type IV collagen gel made at 28°C was contracted and the gel made at 4°C was not at $1,800 \times g$, either the filament diameter of the meshwork would be wider or the densely packed regions would be greater due to the lateral association of the type IV collagen in the meshwork formed at 28°C. Turbidity increase of type IV collagen solution upon incubation at an elevated temperature has been regarded as due to aggregation of the molecules, particularly lateral association (*1, 3, 5, 12*). The turbidity of the gel made at 28°C was higher than that of the gel made at 4°C (data not shown), suggesting that light scattering of the gel formed at 28°C is greater, presumably due to a greater fluctuation of electron density in the three-dimensional space caused by laterally associated portions in the gel meshwork. By contrast, turbidity of the bovine lens capsule type IV collagen gel formed at 4°C or in guanidine-HCl and dithiothreitol was little changed from that of the solution. This may indicate that type IV collagen molecular distribution in the solution changed little upon network formation with repressed lateral association.

If the TH domain of the type IV collagen shares similar properties to those of type I collagen, the TH domains in the meshwork would be highly hydrated at a low temperature under physiological conditions. The strong hydration of TH domains would result in increased partial specific volume of the solute. Thus, the high hydration would increase the buoyant force as well as the frictional force against sedimentation under centrifugal force. Namely, high hydration might be the cause of the high rigidity of gel. Then, what kind of intermolecular interactions would be responsible for the meshwork formation at 4°C? We would assume that interactions involving domains other than the TH domains of the type IV collagen molecule, such as the NC1 domains, might play important roles. We previously suggested that the gel formation of type IV collagen in guanidine-HCl and dithiothreitol might be driven by the domain(s) other than the triple-helical region (*7*).

In contrast to the type I collagen which interacts essentially through the triple helical domains, the type IV collagen molecule contains potentially three different domains to interact with another domain in a neighboring type IV collagen molecule; the triple helical domain (TH), the NC1 domain, which is a noncollagenous globular domain, and the 7S domain which is triple helical and rich in cysteine residues. Among the possible interdomain interactions, four modes of interdomain interactions were reported to exist: (1) TH-TH, (2) NC1-NC1, (3) TH-NC1, and (4) 7S-7S (*5, 12, 13*). Of these four interactions, only the TH-TH interaction was reported to be thermally driven and the temperature dependency of the other three interactions is not well established.

The Pt/C replica of the lens capsule type IV collagen meshwork formed in 20 mM phosphate, pH 7.3, and 150 mM NaCl either at 4 or 28°C showed a fine three-dimensional meshwork structure in the transmission electron micrographs. No distinct difference was observed in the gel meshworks with different rigidities. We assume that the electron microscopic image can not reveal a small difference in the filament diameter, which may reflect the lateral associations, owing to a methodological limitation, *i.e.*, the platinum particle size. The average interval length along the meshwork cord between the branching points was about 10-20 nm in the meshwork. Interestingly, the meshwork structure resembled the basement membrane skeletal structure *in vivo* (*14-16*). On the other hand, the meshwork structure formed in guanidine-HCl and dithiothreitol showed a rather regular arrangement with a honeycomb-like structure when the protein aggregate was examined after guanidine-HCl was washed out (Adachi, E. *et al.*; unpublished observation). Further work will be needed to clarify the similarity and differences in the skeletal structures among these type IV collagen aggregates and basement membrane meshworks.

Kleinman *et al.* reported that the type IV collagen extracted from EHS tumor, together with other basement membrane components, gelated under physiological conditions but that the type IV collagen alone did not. The type IV collagen comprised 30% (dry weight) of the macromolecular components of the gel made from the extract of the EHS tumor tissue (*6, 9*). The apparent inconsistency between the reports on the formation of type IV collagen gel could be attributed to differences in the type IV collagen preparation; the source of type IV collagen (animal species

or tissues) and/or the method for extraction. However, we speculate that essential differences might exist in the protein structure including polypeptide sizes, which could yield a difference in aggregate structures, since the aggregation is the consequence of intermolecular interactions of the type IV collagen. In fact, the lens capsule type IV collagen contained a short α1(IV) chain [160 K α1(IV)] in about an equal amount to the 180K chain, while EHS type IV collagen contained only a 185K-size α1(IV) polypeptide and no short chains (Fig. 1) (*4-8, 17, 18*). In addition, the type IV collagen in the lens capsule acid extract contained tetramer and higher polymers. Since the EHS type IV collagen was extracted from the lathyritic tumor mouse, which was treated with β-aminopropionitrile, an inhibitor of lysyl oxidase-mediated crosslinking, the EHS type IV collagen polypeptides might contain less intermolecular crosslinking. Thus, we believe that the shorter chains and/or higher polymers, which are not contained in the EHS type IV collagen preparation, but are present in a large quantity in the lens capsule type IV collagen preparation, would give rise to the intermolecular interactions which eventually result in the formation of network aggregates in a gel form with more frequent branchings. The distance between branching points of the aggregates, about 15 nm, was smaller than that of the EHS type IV collagen meshwork, reported to be about 40 nm (*5, 19*).

In conclusion, the present report indicated that the lens capsule type IV collagen aggregated into a gel at physiological pH and ionic strength, suggesting that the type IV collagen alone has the potential to form the skeletal meshwork of basement membranes. The mechanical rigidity of the type IV collagen gel was highest at physiological pH and NaCl concentration at 4°C, where type I collagen remains dispersed, suggesting that the type IV collagen has a unique intermolecular interaction.

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