Corneal endothelial cells in culture synthesize basement membrane collagen and secrete it into the medium. This collagen sediments faster than interstitial collagen by velocity sedimentation and is disulfide-bonded. After reduction, two biochemically distinct chains can be determined by cyanogen bromide peptide mapping. These chains migrate close to each other and immediately below β1(1) components on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Treatment with pepsin gives rise to a major band which still retains interchain disulfide bonds and which will not convert to components with the mobility of interstitial α chain by reduction. However, an α chain and three minor collagenase-sensitive and pepsin-resistant peptides are generated if the molecule is reduced and alkylated under nondenaturing conditions prior to pepsin treatment. When collagen which accumulates on the media over a long period of time is compared to the newly synthesized molecules, there is an apparent differential resistance to limited pepsin treatment. However, the products which are generated in both cases share electrophoretic identity.

Basement membranes are specialized connective tissue structures which underlie epithelia and endothelia and which may play a significant role in polarizing the associated epithelial or endothelial cells. These structures are found in the renal glomerulus, lens capsule of the lens, Descemet's membrane of the cornea, and Reichert's membrane of the rat parietal yolk sac as well as other connective tissues (1).

Biochemical analyses have revealed that collagenous proteins are prominent constituents of the basement membranes. Kefalides (2, 3) and his colleagues isolated an α1 chain from pepsin digests of lens capsule and glomerulus and proposed that basement membranes contain a genetically distinct collagen with chain composition [α1(IV)]. Recently, two distinct α chains called C and D chains have been isolated from pepsin digests of anterior lens capsule, glomerular, and placental membranes (4-7). Sato and Sprio (8) found in glomerular basement membrane a heterogeneous mixture of collagen components ranging from 4 = 25,000 to 205,000 which they extracted in the absence of proteases. Bornstein and co-workers (9, 10) characterized type IV procollagen from human amniotic cells in culture which contains two biochemically distinct chains which migrate between β components and pro α1(1) chain. Procollagen molecules of similar size were isolated from anterior lens capsule (11-13). Timpl et al. (14) isolated basement membrane collagen-like materials considerably larger than α chains from EHS tumors not subjected to protease treatment.

Although there is no universally acceptable molecular model for basement membrane collagen, the variously described materials share common characteristics distinct from interstitial collagens. They have elevated but variable levels of 3-hydroxyproline, elevated ratios of 4-hydroxyproline to proline and hydroxylysine to lysine, and low alanine and arginine contents. In addition, hydroxylysine is highly glycosylated with galactose or glucosylgalactose.

The in vitro biosynthesis of basement membrane collagen has been demonstrated in several systems (9, 10, 15-17). The corneal endothelium is a single layer of cells covering Descemet's membrane on the most posterior surface of the cornea of the eye. In this paper, we report the synthesis of basement membrane collagen by corneal endothelial cells in culture and present data indicating that these cells produce two high molecular weight collagenous polypeptides which can in turn generate an α chain like product.

MATERIALS AND METHODS

Culture of Rabbit Corneal Endothelial Cells—Isolation and establishment of corneal endothelial cells in culture were previously described (18). Primary cultures, which were maintained in DME medium (Gibco) supplemented with 10% fetal calf serum (Gibco) and 50 μg/ml of gentamicin, were used throughout the experiment. All cultures were incubated at 37 °C under a humidified atmosphere of 7.5% CO2 in air. Cells grown to confluence in 100-mm plastic culture dishes (Falcon) were labeled for 24 h with 10 ml of DME medium containing 500 μCi of [5-3H]proline (27 Ci/mM) (Amersham) or 100 μCi of [14C]lysyl (371 Ci/mM) (Amersham), 2% fetal calf serum, 64 μg/ml of 2-amino propionitrile, and 100 μg/ml of ascorbate. Medium was collected and the following protease inhibitors were added: 1 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide, 4 mM ethylene-dinitrotolesuccic acid, and 0.1 mM a,a'-dipyridyl. Ammonium sulfate was added to the medium to 45% of saturation after centrifugation

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for removal of cell debris. The precipitate was collected by centrifugation at 10,000 x g for 15 min at 4 °C and dissolved in 2.0 M urea and Buffer I (0.05 M Tris-HCl, 0.15 M NaCl, and 0.1% Triton X-100, pH 7.4) followed by dialysis into the same buffer.

Velocity Ultracentrifugation—The sample was layered on a 5 to 20% sucrose cushion and sedimented in a Beckman SW 40 rotor at 40,000 rpm for 48 h at 4 °C. The gradient was then fractionated. Fractons containing collagenous proteins were pooled and dialyzed against 6.0 M urea in Buffer I at 4 °C. The samples were denatured by heating to 55 °C for 30 min and sedimented on a 5 to 20% sucrose gradient containing 6.0 M urea and Buffer I in a Beckman SW 40 rotor at 40,000 rpm for 48 h at 20 °C.

SDS-Polyacrylamide Slab Gel Electrophoresis—Polyptides were electrophoresed under the conditions described by Laemmli (19) and fluorograms were developed by Kodak RPX-Omat processor (20, 21).

Peptide Mapping by CNBr Digestion—CNBr digestion was carried out as described previously (22) with some modifications. Protein bands localized in slab gels by fluorography were cut out of the dried gel. These were rehydrated in 70% formic acid and digested with 1.0 M CNBr for 4 h at 30 °C. At the end of the digestion, the gel pieces were dried down in a water bath under bubbling Nz gas and washed with distilled water three times. They were then placed into the slot of a 3% stacking gel and electrophoresed on a 10% running gel.

Analysis for Hydroxyproline—The hydroxyproline content of the sample was determined after hydrolysis in 6 M HCl under vacuum at 110 °C for 24 h. The hydrolysate was dried and then dissolved in 0.01 M HCl. It was then applied to a short column of a JEOI amino acid analyzer (JLCdAH). Elution was carried out with 0.2 M sodium citrate buffer, pH 2.81, at 35 °C. Each fraction was counted in a counter.

Reduction and Alkylation under Nondenaturing Conditions—Partially purified sample from sucrose velocity sedimentation was dialyzed against 0.1 M Tris-HCl, 0.15 M NaCl, and 2.0 M urea, pH 7.5, at 4 °C and dialyzed. Dithiothreitol (final concentration, 20 mM) was added and the reaction mixture was stored for 4 h at 4 °C. Iodoacetamide (final concentration, 80 mM) was then added followed by incubation for 24 h at 4 °C.

Enzyme Digestions—Samples were incubated with 100 μg/ml of pepsin for 24 h at 4 °C or 24 h at 15 °C or 22 °C. Pepsin activity was stopped by raising the pH to 8.0 by addition of solid Tris and boiled for 1 min in the presence of 0.2% SDS. Protease-free bacterial collagenase digestion was performed as described by Peterkofsky and Diegelman (23). The reaction was stopped by addition of SDS to a final concentration of 0.2% followed by boiling.

Preparation of Marker Collagen—Fibroblasts from embryonic rabbit skin were cultured by conventional techniques (24). Secondary cultures were then labeled and the collagen purified (24).

RESULTS

Isolation and Purification of Basement Membrane Collagen—Basement membrane collagen synthesized by the corneal endothelial cells was recovered from the medium. Collagenous proteins accounted for approximately 12 to 15% of the total nondialyzable [3H]proline-labeled protein. Initial isolation was performed by ultracentrifugation under native conditions. A typical sedimentation profile is shown in Fig. 1A. Brief preincubation of the ammonium sulfate precipitate prior to sedimentation with bacterial collagenase partially removed peak I and almost completely peak II. Peak I sedimented faster than a type I collagen marker (Fig. 1A). Limited pepsin treatment of peak I material rendered a disulfide-linked collagen reducible to one α1 size band (data not shown). These results suggest that peak I may contain a precursor form of type III collagen (Fig. 1B, arrow). Peak II is rich in two closely spaced collagenous bands migrating immediately below β components and fibronectin. These were designated as CE-1 and CE-2. Similar migrating bands have been reported in human amniotic fluid cell culture systems (9, 10). One of the components of peak III was identified as fibronectin by immunoprecipitation technique (antibody against fibronectin was provided by Dr. Erkki Ruoslahti, La Jolla Cancer Research Foundation, La Jolla, CA).

Fig. 1. Velocity sedimentation under native conditions and fluorograms. A, velocity sedimentation of [3H]proline-labeled medium proteins under native conditions. Ammonium sulfate precipitate from the medium proteins was sedimented in a Beckman SW 40 rotor at 40,000 rpm for 48 h at 4 °C. Type I collagen (open arrow) was sedimented in the companion tube at the same time. B, electrophoretogram of fractions in A on 4.5% SDS-polyacrylamide gel electrophoresis under reduced conditions. M, collagen marker; FN, fibronectin; CE-Ⅰ and CE-2, basement membrane collagen; NCP, noncollagenous protein. C, electrophoretogram of fractions isolated by velocity sedimentation of [35S]cystine-labeled medium proteins on 4.5% SDS-PAGE under reduced conditions. M, collagen marker; NCP, noncollagenous protein; CE-1 and CE-2, basement membrane collagen.
The materials from peak II were pooled and further purified by ultracentrifugation under denaturing conditions. The sedimentation profile of the denatured CE collagen was compared with type III collagen. Denatured type III collagen whose molecular weight is about 300,000 sedimented to fraction 25, while denatured CE collagen sedimented much faster (Fig. 2).

Type III collagen contains intrahelical disulfide bridges near the carboxyl-terminal end (25, 26). The unusually fast sedimentation of denatured CE collagen suggests that in addition to its larger molecular size, it may represent a more compact structure than type III collagen. When fractions 36 and 37 were analyzed on SDS-PAGE under reducing condition, the characteristic CE-1 and CE-2 bands were the only peptides present, suggesting that this is a good method for purification.

When cystine-labeled medium fraction was analyzed on SDS-PAGE after ultracentrifugation, noncollagenous, pepsin-sensitive, proline-poor, and cystine-rich protein co-sedimented with CE collagen. This molecule migrated to the position of \( \alpha_1(I) \) prior to reduction (not shown). However, after reduction, it migrated slower (Fig. 1C, NCP).

**Characterization of the Basement Membrane Collagen—**

When the materials from peak II in Fig. 1A were incubated with pepsin at 4 °C or at 15 °C for 24 h, the electrophoretic mobility of the major collagenous protein was unaltered (Fig. 3A, inset, lanes 3 and 4). Reduction often generated a heavy band which migrated just below standard CE-1 and a very faint band just below CE-2 (Fig. 3A). Although this treatment also generated a low molecular weight fragment (Fig. 3A, inset, arrow), there was no conversion of the basement membrane collagen to components with the mobility of interstitial \( \alpha \) chains. When the materials from peak II were reduced and alkylated under nondenaturing conditions, the reduced and alkylated molecules also failed to show a change in the electrophoretic mobility (Fig. 3B). However, pepsin digestion of the reduced and alkylated molecules generated an \( \alpha \) chain and three low molecular weight collagenase-sensitive fragments (Fig. 3B). In order to study whether the \( \alpha \) chain has pepsin-sensitive sites and is able to generate these low molecular collagens fragments, prolonged digestion at a high enzyme to substrate ratio and at elevated temperatures (15 °C and 22 °C) was used. An essentially identical profile of collagenous peptides was recovered under all experimental conditions tested (Fig. 4).

Since one of the characteristics of basement membrane collagen is that of a elevated ratio of hydroxyproline to proline, the extent of hydroxylation of \([3H]\)proline was determined.

**Fig. 2.** Velocity sedimentation under denaturing conditions. Peak II in Fig. 1A was denatured and sedimented as described in the text. Inset, electrophoretogram of fractions 36 and 37 on 4.5% SDS-PAGE after reduction. Denatured type III collagen (arrow) was sedimented in the companion tube at the same time.

**Fig. 3.** Densitometric scans of fluorograms and fluorograms of peak II materials in Fig. 1A. A, inset, peak II materials resolved by 4.5% SDS-PAGE under the nonreduced condition. Lane 1, collagen marker; Lane 2, peak II materials after bacterial collagenase digestion; lane 3, peak II materials; lane 4, peak II materials after pepsin treatment, 100 \( \mu \)g/ml, 4 °C, 24 h. Graph A, peak II materials after pepsin digestion resolved in 4.5% SDS-PAGE under the reduced condition. B, ———, reduced and alkylated peak II material; ———, pepsin digests of the reduced and alkylated peak II material.

**Fig. 4.** Densitometric scans of 5% SDS-PAGE fluorograms. Reduced and alkylated peak II materials were digested with pepsin (200 \( \mu \)g/ml, 24 h). ———, at 15 °C; ———, at 22 °C. The major peak migrated to \( \alpha_1(I) \) marker.

Samples from the various steps were analyzed (Table I). Peak II material in Fig. 1A showed 36% hydroxylation, whereas the same sample followed by pepsin digestion showed 45%. When peak II was analyzed after reduction and alkylation under nondenaturing conditions, approximately 42% of the nondi-
alalyzable $^3$H activity was recovered as hydroxy[$^3$H]proline. The sample recovered by the ultracentrifugation under denaturing conditions contained about 43% of its imino acid as hydroxy[$^3$H]proline.

In order to study whether CE-2 might be a proteolytic degradation product of CE-1, both polypeptides were separately digested with cyanogen bromide. The resulting peptides were resolved by electrophoresis. Since major CNBr peptides from CE-1 (Fig. 5, arrows) are not present in CE-2 peptides, this excludes the possibility that CE-1 and CE-2 have common origins.

Since pepsin treatment of the newly synthesized CE collagen fails to generate components with the mobility of interstitial $\alpha$ chains, it seemed of interest to examine the characteristics of CE collagen accumulated in the media over a long period of time. Confluent endothelial cells were kept in culture for 13 days in the presence of [$^{14}$C]proline. On the 13th day,
Rabbit corneal endothelial cells in culture synthesize and secrete mostly basement membrane collagen (CE) and a precursor form of type III collagen as a minor component. The electrophoretic behavior and the degree of susceptibility to limited pepsin treatment of CE collagen are similar to AF, procollagen synthesized by human amniotic fluid cells (9, 10) and endothelial cells (27, 28). Like these collagens, CE collagen migrated as distinct chains just below collagen β components upon reduction. Pepsin treatment yields one or two chains which migrate just below the position of CE collagen upon reduction (Fig. 3). Although CE collagen appears as a doublet on SDS-PAGE after reduction, the ratio of the two bands, CE-1/CE-2, is much greater than 2 but fluctuates with the conditions of purification.

The triple-stranded disulfide-linked CE collagen can be purified by velocity ultracentrifugation, and comparison of the sedimentation profiles under denaturing conditions with type III collagen demonstrates its faster sedimenting behavior. This may be attributed to two factors: 1) the larger molecular size of CE collagen compared to collagen, and/or 2) a more globular (or compact) conformation of CE collagen due to disulfide bonds at both ends (probably involving noncollagenous extensions).

It is of interest to note that a noncollagenous, pepsin-sensitive, proline-poor, and cystine-rich protein (Fig. 1C, NCP), whose molecular weight is approximately 100,000 as determined by electrophoretic mobility, co-sedimented with CE collagen. This NCP is heavily labeled by cystine and appears to contain intramolecular disulfide bonds which upon cleavage generate a molecule of larger hydrodynamic volume. This observation suggests two possibilities. 1) This NCP may form aggregates as judged by its sedimentation behavior and these aggregates sedimented coincidentally with CE collagen; and/or 2) this NCP may be associated with this collagen and explain the different recovery and inconsistent appearance of the two closely spaced chains of CE collagen when an ammonium sulfate precipitate was examined on SDS-PAGE.

The extent of hydroxylation of proline residues in CE collagen is lesser than other basement membrane collagens. This difference may suggest three possibilities. 1) CE collagen has a low degree of hydroxylation; 2) underhydroxylation may be associated with the tissue culture conditions; and/or 3) other extensions or modifications may protect the molecule from enzymatic attack, thus, allowing CE collagen to retain noncollagenous extensions. Therefore, the hydroxyproline content becomes diluted by these noncollagenous sequences.

On the other hand, the fact that highly purified CE collagen by pepsin treatment or from the reduced and alkylated material followed by pepsin treatment contains low hydroxyproline values seems to rule out the latter possibility. The substantial differences in CNBr peptide profiles rule out the possibility that CE-2 might originate from a common precursor.

Dehm and Kefalides (3) generated an α size chain from bovine lens capsule by a procedure which includes two-step proteolysis by pepsin. The collagen is indistinguishable from α(II) chain on SDS-PAGE. Miller and other groups (4-7) reported two biochemically distinct chains, C chain (α size) and D chain (80,000) differing in ionic charges and CNBr cleavage patterns. On the other hand, larger peptides which migrate just below β components on SDS-PAGE after reduction were recovered from the several tissue culture systems (9, 10, 27). The present study provides evidence that an α like chain is derived from the high molecular weight chains which migrate just below β components. Limited pepsin digestion of the intact CE collagen does not convert the molecule to components with the mobility of interstitial α chains, and it seems that reduction and alkylation prior to pepsin digestion are necessary to generate such a product. It has been reported that an α size chain can be isolated from lens capsule subjected to partial limited proteolysis before and after reduction under non denaturing conditions (3). Therefore, it is clear that partial reduction is necessary for the enzyme to cleave the basement membrane collagen into α size chains.

Since newly synthesized CE collagen does not respond to pepsin in the same manner as interstitial procollagens, the reasons were further investigated. When newly synthesized CE collagen was labeled with [3H]proline for 24 h, the ratio of the resulting peptides after pepsin treatment, CE-1p/CE-2p, varies. In all cases, however, the intensity of CE-1p is much greater than that of CE-2p, suggesting not only that CE-1 and CE-2 are distinct molecules but that the newly synthesized CE-2 is much more sensitive to limited proteolysis. On the other hand, when CE collagen was labeled to accumulate for 13 days in culture, the CE-1p and CE-2p were recovered in similar amounts (Fig. 6, lane 3). The fact that the stoichiometry of the CE-1 and CE-2 chains is greater than 2:1 and that they exhibit a differential susceptibility to limited proteolysis is further evidence that CE-1 and CE-2 are distinct species. One can speculate that the α size chains obtained from pepsin digestion of the reduced and alkylated peak II materials (Fig. 3B) may be derived from CE-1 (in contrast to the small molecules which probably originate from the pepsin unstable CE-2). Although further evidence is needed to support this speculation, single basement membrane collagen species containing two chains cannot be ruled out.

It has recently been reported that type III collagen was the major collagenous component in bovine corneal endothelial cell culture (29). Our discrepancy with these findings may be due to the culture age (primary versus late passages), animal species (rabbit versus bovine), or the culture conditions (absence versus presence of growth factors). It should be noted that type III collagen in the bovine system was determined by only DEAE- and carboxymethyl (CM)-cellulose column chromatography, while electrophoretic mobility, sedimentation behavior, and CNBr peptide patterns were used in the present study. Rabbit corneal endothelium in organ culture incubated with radioactive precursor synthesizes only basement membrane collagen (16). However, type III procollagen is synthesized as a minor collagenous component in rabbit corneal endothelial cells in culture. Immunofluorescent studies with antitype IV antibody (gift of Dr. George Martin, National Institutes of Health, Bethesda, MD) and anti-type III antibody show that rabbit Descemet's membrane only stains with anti-type IV antibody.7 Presently, it is not clear whether type III collagen is a component of Descemet's membrane.

These results may provide a link between the two electrophoretically distinct species of basement membrane collagens described in the literature, namely CE-1 and CE-2 collagen, AF3, procollagens, and C and D chain.

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