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Isolation and Characterization of Human Placental Chorionic Villar Extracellular Matrix

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The cell-free extracellular matrix of human placental chorionic villi has been prepared by a procedure employing extraction of the terminal villar fragments with the detergents Triton X-100 and sodium deoxycholate. The isolated human placental extracellular matrix retains an intact, but collapsed, histoarchitecture, as observed by scanning and transmission electron microscopy. It remains intact, in large part because of the presence of continuous sheets of villar basement membranes and associated interstitial collagen fibers and scattered patches of fibrin. The staining characteristics and chemical composition of the isolated human placental extracellular matrix are similar to those reported for basement membranes in several tissues and indicate the presence of collagen-like and glycoprotein components in this preparation. Gel electrophoresis of urea-SDS-mercaptoethanol extracts of the matrix showed that it consists of several polypeptide components of various molecular weights, some of which are associated into high molecular weight complexes by disulfide bonds.

Key words: human placental basement membrane, extracellular matrix, human chorionic villar basement membrane

The human placental chorionic villi compose a continuous barrier between the blood of the mother and the fetus and provide a large surface area for the exchange of gases, nutrients, and drugs across this barrier. An essential component of this exchange surface is an extracellular matrix comprising connective tissue components, including the basement membrane, which may have both support and permeability functions in the villi. Despite the general interest in the structural and permeability properties of vascular basement membranes in health and disease, only a few limited studies have been attempted on the isolation and characterization of trophoblast basement membrane from human chorionic villi [Gang and Gelfand, 1971; Schwartz et al, 1974; Bray et al, 1975; Gutman et al, 1977]. These studies have in common the use of a combination of sieving and sonication techniques to obtain the trophoblast basement membrane and associated connective tissue components.

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Since the chorionic placental villar basement membrane is intimately associated with interstitial striated collagen fibers, fibrin, and other possible connective tissue components [Verbeek et al, 1967], employment of techniques analogous to those used in the isolation of glomerular basement membrane [Spiro, 1967], which is not associated with such contaminants, would not be expected to yield a pure basement membrane fraction. Indeed, the placental membrane fractions isolated in these studies did not resemble basement membranes isolated from other tissues in amino acid composition [Meezan et al, 1978; Carlson et al, 1978], being markedly less collagenous in character. Since striated interstitial collagen fibers would be expected to be a major component, together with basement membrane, of human chorionic villar extracellular matrix [Verbeek et al, 1967], the noncollagenous nature of the material isolated by these groups did not seem to be in accordance with the known chemical composition of these components. We have, therefore, reinvestigated the isolation of human chorionic villar extracellular matrix [Brendel et al, 1978] using a combination of sieving and detergent extraction techniques that have proved useful in the study of basement membranes and associated extracellular matrix components in other tissues [Meezan et al, 1975; 1978; Carlson et al, 1978; Meezan et al, 1979]. Our results indicate that human chorionic villar extracellular matrix consists of basement membrane associated with significant amounts of collagen and fibrin, and that this material has a chemical composition that is distinctly collagenous in nature and that resembles basement membranes obtained from other tissues [Meezan et al, 1978; Carlson et al, 1978].

MATERIALS AND METHODS

Collection of Placental Villi

Normal human placentas from full-term pregnancies were stored at 4°C immediately after delivery. Placentas were quickly cleaned in running water and immersed in a solution containing 10 mM EDTA, 0.9% NaCl at pH 7.0. After removal of the umbilical cord and trimming off of remnants of the amniotic sac and other loose debris in the buffer, placentas were squeezed softly several times in fresh EDTA-saline buffer to remove blood and blood clots. At this time the color of the cotyledons became pink, from an initial dark red. With a metal spoon, the terminal villi were obtained by careful scraping of the cotyledons from the maternal side of the placenta into a large beaker containing 0.9% NaCl, 10 mM EDTA solution. The terminal villi obtained were diluted with saline EDTA buffer to 900 ml and blended in an ordinary home blender at low setting for 10 sec. The mixture was then diluted to 3 liters with saline EDTA buffer and washed through a large-size 1 mm^2 gauge nylon sieve. The material on the sieve was resuspended in 2 liters of the saline EDTA buffer and sieved again. The material remaining on the sieve consisted of large vessels and unbroken vascular trees and was discarded. The material passing through the coarse sieve was collected and extensively washed on a 110 μm gauge nylon sieve. At this stage, the material was light pink in color. The filtrate was discarded. The material on top of the sieve was transferred back to the blender and blended again, this time at the high setting for 30 seconds. The suspension was then diluted to 3 liters and passed through a 210 μm gauge nylon sieve to remove remaining vascular contaminants. The purified terminal villi in the filtrate were collected on a 110 μm mesh sieve again and washed thoroughly with water to partially lyse cells and remove remaining blood components. This villar fraction was the starting material for the isolation of the extracellular matrix.

Isolation of Villar Extracellular Matrix

The collected terminal villi from 9 human term placentas of approximately 200 gm wet weight were suspended in 4 liters of distilled water containing 1 mM EDTA, stirred, and allowed to settle. The supernatant was then carefully suctioned off. This process was repeated five times. This procedure helps in the removal by osmolysis of cells that have resisted the previous washings on the sieve. The suction procedure, rather than ordinary decantation, minimizes the loss of small villi. At this stage, when viewed under a stereomicroscope, the sample of terminal villi should appear essentially homogeneous. To the last suspension was added an amount of 10% Triton X-100 containing 0.5% NaN_3, to adjust the final concentration to 1% Triton and 0.05% NaN_3, and this suspension was gently stirred by a magnetic stirrer for three hours. After settling for 30 min, the somewhat cloudy supernatant was suctioned off. Fresh 1% Triton solution was added, and the suspension was stirred overnight. After sedimentation, most of the supernatant was aspirated off and new Triton added. This suspension was blended on the high setting for 5 sec. The suspension was then transferred to a sieve with an 80 μm gauge and extensively washed with distilled water until foaming could no longer be observed. The wet material on the sieve was transferred to 1 liter of 0.1 M acetate buffer at pH 7.5 containing 10 mM $CaCl_2$, 0.05% NaN_3, and 0.5 mM phenylmethylsulfonylfluoride (PMSF) to which 4,000 Kunitz units of DNAase II (spleen) was added and stirred for 3 h. The cell-free placental villi were then washed again on the sieve, first with 1 M NaCl solution, then with distilled water. At this stage, the material was transferred to 500 ml of 4% sodium deoxycholate solution and stirred for 4 h. After this time the material was centrifuged and the pellet resuspended in water and recentrifuged 3 times, followed by resuspension and recentrifugation in 20%, 40%, 60%, 40%, and then 20% methanol. Finally, the material was extensively washed with distilled water on a 64 μm gauge sieve, collected, and lyophilized.

Microscopy and Analytical Procedures

Light, phase-contrast, scanning and transmission electron microscopy of isolated villi and extracellular matrix preparations were carried out by previously described procedures [Carlson et al, 1978; White et al, 1979]. Amino acid analysis was done on a Beckman 121 C amino acid analyzer according to the modification of Guire et al [1974] with samples that had been hydrolyzed with constantly boiling 6 N HCl in sealed glass tubes under nitrogen for 22 h at 110°C. Neutral and amino sugars were quantitated by gas chromatography according to a method modified from that of Grimes and Greeger [1976] as described previously [Carlson et al, 1978]. Sialic acid was determined by the method of Warren [1959]. Sodium dodecylsulfate polyacrylamide gel electrophoresis was performed as described by Weber and Osborn [1969], with staining and destaining according to the method of Fairbanks et al [1971]. Samples (1 mg) were dissolved in 0.4 ml of 6 M urea, 2% SDS, 0.1 M phosphate buffer (pH 7.0) with and without mercaptoethanol (10 μl) by stirring overnight at room temperature.

Triton X-100 was purchased from Mallinckrodt Co. DNAase (bovine spleen, type II) was obtained from Sigma. All reagents for gel electrophoresis were obtained from Bio-Rad. Nylon bolting cloth of various gauges was obtained from Tetko, Inc. Urea was purchased from Aldrich Co. and recrystallized from water at pH 4.0 in order to eliminate isocyanate. All other chemicals were reagent grade of best available commercial quality.

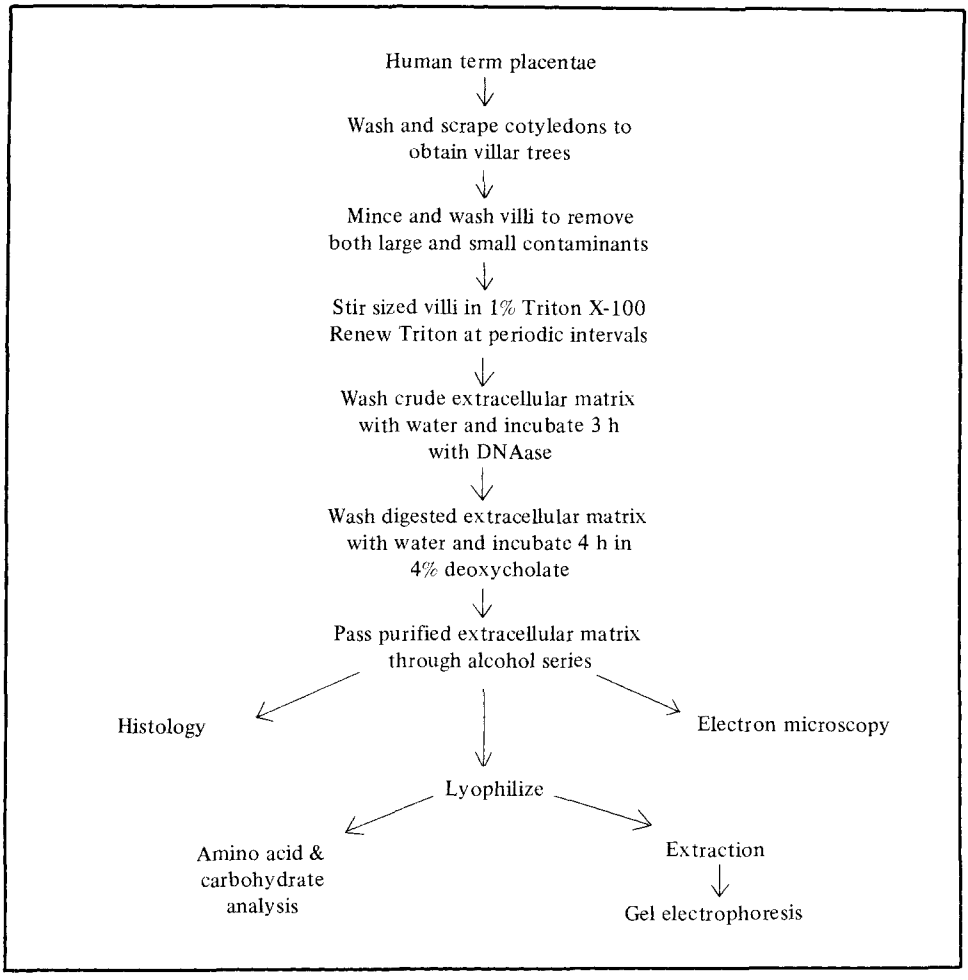

Fig. 1. Flow chart of human placental villar extracellular matrix isolation and characterization. See Methods for details.

RESULTS

Isolation Procedures

The isolation scheme for human placental chorionic villar extracellular matrix is outlined in Figure 1. The blending and sieving procedures employed in this study yielded a preparation of terminal chorionic villi that was generally free of large-vessel contamination and that appeared to be made up of multibranched objects (Fig. 2a) by phase contrast microscopy. After detergent treatment, this material assumed an almost transparent, folded, sheet-like appearance in the phase-contrast microscope, reminiscent of that obtained by similar treatment of isolated renal glomeruli (Fig. 2b). Scanning electron microscopy of intact human placental terminal villi confirmed the smooth, finger-like arrangement of the

villar surfaces (Fig. 2c), which was transformed into a folded, sheet-like arrangement of large surface area comprising the total extracellular matrix of these structures following detergent treatment (Fig. 2d). Transmission electron microscopy of intact human placental villar microvessels in situ revealed an arrangement of blood vessels bounded by a smooth, continuous basement membrane with adjacent, closely associated interstitial collagen fibers also present (Fig. 2e). After complete detergent treatment to obtain the human placental villar extracellular matrix, the boundary histoarchitecture of the villi was largely maintained as a skeleton of amorphous basement membrane material with numerous associated and enclosed interstitial striated collagen fibers (Fig. 2f). The preparation was free of intact cells, nuclei, and cellular debris, as determined by staining with periodic acid-Schiff and eosin-hematoxylin stains. Staining with the Jones silver stain revealed ribbons of black basement membrane material. Patches of fibrin-like material were observed after staining with phosphotungstic acid-hematoxylin and upon examination of grids by transmission electron microscopy. The final yield of lyophilized purified extracellular matrix is usually 0.5–1.0 gm of material per placenta.

Chemical Analyses

The amino acid and carbohydrate analyses of isolated human placental chorionic villar extracellular matrix are shown in Table I, together with the analyses reported for human placental membranes isolated by another group [Bray et al, 1975] and those reported for a sample of human renal glomerular basement membrane [Kefalides, 1974]. The composition of the placental extracellular matrix isolated using the procedures in this study was rich in glycine and glutamic and aspartic acids and contained appreciable amounts of proline, hydroxyproline, lysine, and hydroxylysine, all of which indicated it to be collagenous in nature. Virtually all of the hydroxyproline was in the form of 4-hydroxyproline, with only trace amounts of 3-hydroxyproline being detected in the preparation. Carbohydrate analysis revealed galactose and glucose to be present in the greatest amounts with lesser quantities of mannose and glucosamine and only minor amounts of galactosamine, fucose, and sialic acid. Since fucose elutes in a region that, by gas chromatography, contains several foreign peaks, its value represents the maximum possible content of this sugar in the preparation.

SDS Gel Electrophoresis

The gel electrophoresis patterns of human placental chorionic villar extracellular matrix extracted and electrophoresed under nonreducing conditions with SDS-urea and that extracted and electrophoresed in the reduced state with SDS-urea-mercaptoethanol are shown in Figure 3. When the matrix was extracted with 6 M urea containing 2% SDS at room temperature for 24 h and electrophoresed on a 5% acrylamide gel, the bulk of the extracted protein barely penetrated the gel. Of the material that did migrate into the gel, two prominent high molecular weight bands had a mobility similar to that of calf skin collagen β-chains, and the remainder of the material fractionated as one major and several minor staining lower molecular weight components. Similar extraction and electrophoresis under reduced conditions in the presence of mercaptoethanol gave a more complex pattern, with virtually all of the extracted material migrating into the gel and with the appearance of several new, or much more prominent, bands not present or only faintly discernible in the unreduced sample. About one-third of the isolated extracellular matrix was soluble in 6 M urea or 6 M urea–2% SDS alone, and another third was solubilized in the presence of mercaptoethanol.

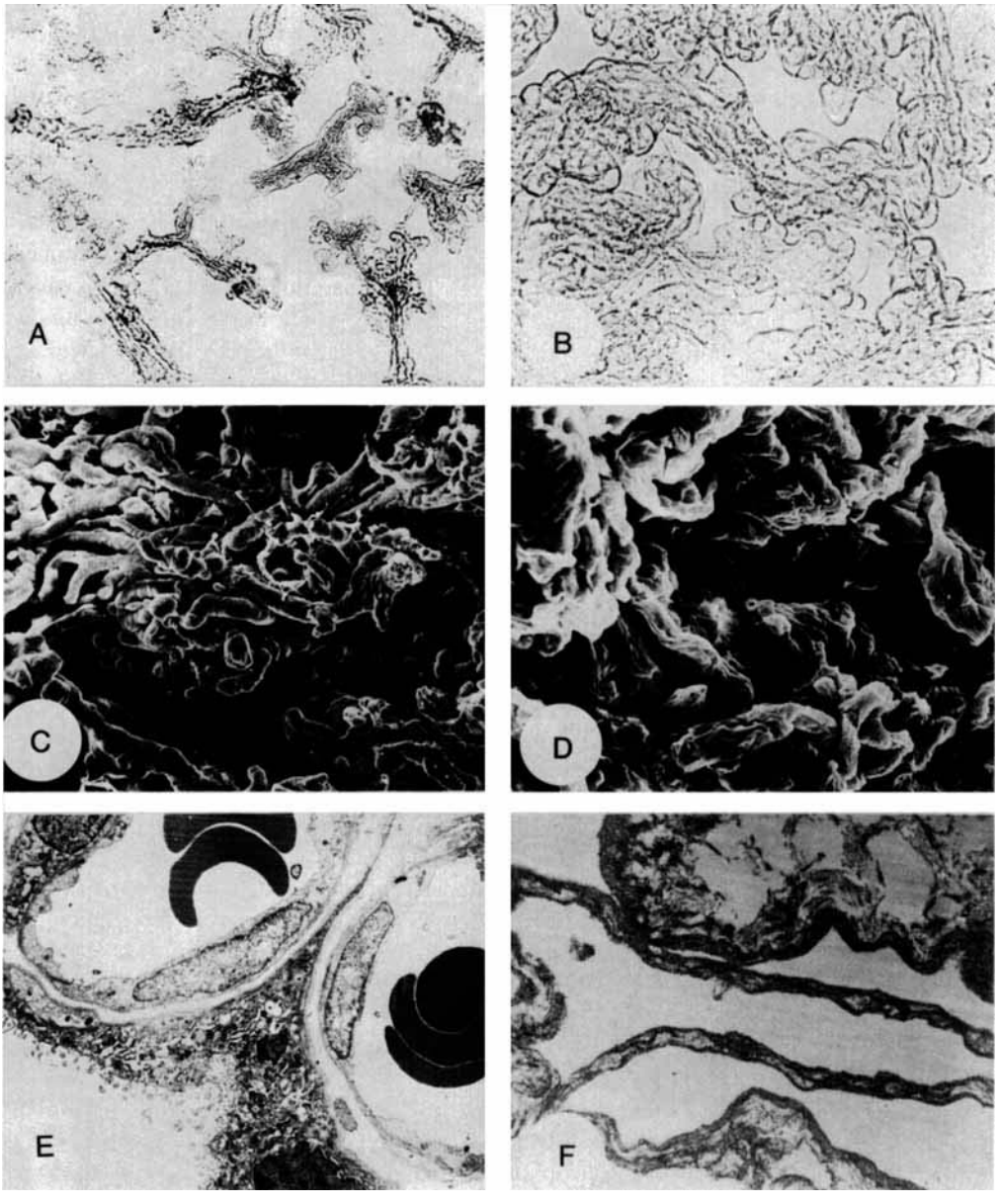

Fig. 2. A, Phase-contrast photomicrograph of isolated terminal chorionic villi (magnification, × 60). B, Phase-contrast photomicrograph of isolated chorionic villar extracellular matrix (magnification, × 150). C, Scanning electron micrograph of intact placental chorionic terminal villi in situ. Note multi-branched, finger-like arrangement of villi similar to that seen in A (magnification, × 100). D, Scanning electron micrograph of isolated placental chorionic villar extracellular matrix. The tube-like arrangement seen in the intact villi in C has collapsed into a folded, sheet-like arrangement after detergent treatment (magnification, × 210). E, Transmission electron micrograph of human placental terminal villar microvessels in situ. Cross-sections of two villar capillaries containing enclosed red blood cells are shown. A thick, continuous trophoblast basement membrane lies immediately adjacent to the cytoplasmic membranes of both syncytio- and cytotrophoblasts (lower left) separated from the capillary endothelium by a connective tissue layer containing collagen fibrils, adjacent to a thin, discontinuous capillary basement membrane (magnification, × 900). F, Transmission electron micrograph of isolated placental chorionic villar extracellular matrix. Although the shape of the villar capillary spaces is not retained after detergent treatment, continuous basement membranes with enclosed and embedded interstitial collagen fibers still border and enclose clear areas that represented fetal capillary and maternal blood spaces. Intact villar or red blood cells are not seen in these preparations (magnification, × 2,100).

TABLE I. Analytical Comparison of Human Chorionic Villar Extracellular Matrix With Human Placental Membranes and Human Glomerular Basement Membrane

	Residues/1,000 amino acids		
Amino acid	Villar extracellular matrix[a]	Placental membranes fraction 1[b]	Human glomerular basement membrane[c]
3-Hydroxyproline	0.4	NR	7.0
4-Hydroxyproline	59.6	18.8	66.0
Aspartic acid	77.5	107.0	65.0
Threonine	36.2	49.6	40.0
Serine	50.5	72.2	60.0
Glutamic acid	97.9	111.0	103.0
Proline	78.9	56.7	62.0
Glycine	213.0	124.0	227.0
Alanine	76.4	58.2	58.0
1/2 Cystine	11.7	22.8	23.0
Valine	40.1	45.0	36.0
Methionine	10.6	17.2	7.0
Isoleucine	30.1	39.7	28.0
Leucine	56.5	67.0	66.0
Tyrosine	17.9	29.3	14.5
Phenylalanine	26.1	28.8	28.0
Hydroxylysine	12.4	4.6	24.5
Lysine	37.1	61.2	26.0
Histidine	14.3	18.3	18.7
Arginine	52.8	50.8	48.3
Tryptophane	NR	17.0	NR
Carbohydrate	μg Sugar/mg matrix		
Fucose	<0.72	34[d]	7
Mannose	5.31		17
Galactose	12.53		26
Glucose	9.98		25
Glucosamine	6.47	13.6[d]	17
Galactosamine	1.25		3
Sialic acid	2.41	6.4	15

[a]Average of 4 amino acid analyses and 9 carbohydrate analyses on a large-scale preparation derived from 9 normal human placentae.
[b]Taken from Table I of Bray et al [1975].
[c]Taken from Tables I and II of Kefalides [1974].
[d]Reported as total hexose and hexosamine.
NR = not reported.

DISCUSSION

The procedure described here for the isolation of human placental chorionic villar extracellular matrix is similar to that developed previously in this laboratory for the isolation of basement membranes from renal glomeruli and tubules and purified retinal and brain microvessels [Meezan et al, 1975; Meezan et al, 1978; Carlson et al, 1978]. However, the material obtained after detergent extraction of the isolated terminal villi is not a purified basement membrane, as one would obtain if the starting material were renal glomeruli, but rather it is an extracellular matrix in which basement membrane, striated interstitial collagen fibers, and patches of fibrin retain the histoarchitectural outlines of the villar microvasculature. Whereas typical striated collagen fibers were reported to be seen only oc-

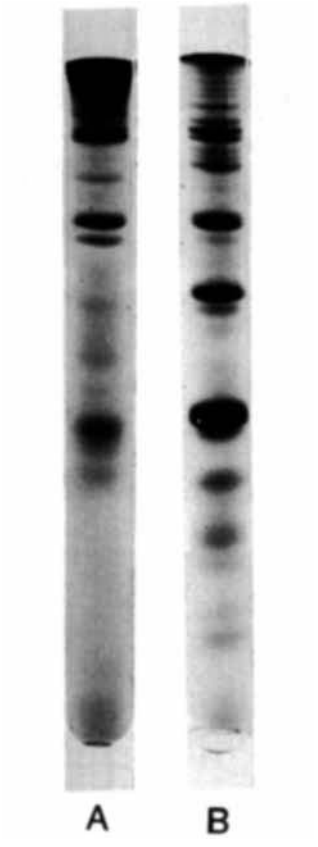

Fig. 3. SDS gel electrophoresis of 6 M urea–2% SDS extract of chorionic villar extracellular matrix electrophoresed on 5% acrylamide gel in the absence of mercaptoethanol (A) and of 6 M urea–2% SDS–2% mercaptoethanol extract (B). See text for details.

casionally in other preparations of chorionic villar membranes [Bray et al, 1975], they were commonly seen closely associated with the basement membranes in our preparations, a finding in agreement with their in situ location in the chorionic terminal villi [Verbeek et al, 1967] used as starting material for the isolation. Likewise, the presence of fibrin could be demonstrated both by histochemical staining and by electron microscopy and probably represents placental fibrinoid as postulated by Bray et al [1975].

Our preparation of human chorionic villar extracellular matrix also differed markedly in chemical composition from preparations of trophoblast basement membrane reported by others [Gang and Gelfand, 1971; Schwartz et al, 1974; Bray et al, 1975; Gutman et al, 1977], being collagenous in nature and remarkably similar to human renal glomerular basement membrane in overall amino acid composition (Table I). In contrast, the amino acid compositions of previously reported preparations of trophoblast basement membrane either lacked hydroxyproline and hydroxylysine completely [Schwartz et al, 1974; Gutman et al, 1977] or contained much lower amounts of these amino acids and glycine than those reported from basement membranes obtained from other vascular and nonvascular tissues [Meezan et al, 1978; Carlson et al, 1978]. In fact, one such preparation of placental membranes [Bray et al, 1975] most closely resembled fibrin in its amino acid composition, immunologic properties, and suceptibility to enzyme digestion. In view of the collagenous

nature of all previously characterized basement membranes obtained from a variety of tissues [Meezan et al, 1978; Carlson et al, 1978], it is possible that the material isolated by previous investigators using screen and sonication techniques represented placental fibrinoid rather than trophoblast basement membrane.

The presence of only trace amounts of 3-hydroxyproline (in our preparations of placental extracellular matrix), an amino acid that usually is enriched in basement membrane collagens, is not surprising in view of the facts that this preparation is not a pure basement membrane and that preparations of collagens believed to be of placental basement membrane origin contain lower amounts of this amino acid than other basement membrane collagens [Burgeson et al, 1976; Rhodes and Miller, 1978]. The carbohydrate composition of our preparation contains the same relative proportion of neutral and amino sugars as that present in preparations of renal glomerular basement membrane [Spiro, 1967], although in lesser amounts, which indicates that glycoproteins are constituents of the placental extracellular matrix.

The gel electrophoretic patterns and solubility behavior of extracts of human chorionic villar extracellular matrix revealed that the membrane consists of several polypeptide components that are associated with one another by disulfide, and possibly other, covalent bonds. About two-thirds of the matrix was soluble by extraction with urea-SDS-mercaptoethanol, but one-third of the matrix remained insoluble even after exhaustive extraction and had a composition similar to that of collagen [Ohno et al, in preparation]. The multiple banding pattern given by placental extracellular matrix extracts on gel electrophoresis was reproducible and was unaltered whether the matrix isolation was carried out in the presence or the absence of proteolytic enzyme inhibitors, indicating that it was not an artifact of degradation during isolation. In this respect it resembles the behavior of isolated renal glomerular basement membrane [Hudson and Spiro, 1972; Sato and Spiro, 1976], in which multicomponent characteristics have been ascribed to in vivo proteolysis [Spiro, 1976], a possibility that must also be considered in the placenta.

Since purified chorionic villar basement membrane cannot be isolated free of significant interstitial collagen and fibrin contamination, as is possible with the basement membranes of several other tissues [Meezan et al, 1975; Meezan et al, 1978; Carlson et al, 1978]. further work in our laboratory will focus on the identification, fractionation, and isolation of basement membrane components from this acellular preparation of placental extracellular matrix.

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