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Simultaneous preparation of paired, syncytial, microvillous and basal membranes from human placenta

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A method for the simultaneous preparation of microvillous and basal membrane vesicles from human placental syncytiotrophoblast is described. Mg^{2+}-aggregated basal membranes are separated from microvillous membranes by low-speed centrifugation after initial homogenization and centrifugation steps. Microvillous membranes (MVM) are obtained from the low speed supernatant while basal membranes (BM) contained in the Mg^{2+}-aggregated material are resuspended and further purified on a sucrose step gradient. MVM and BM prepared by this method were enriched 20-fold and 11-fold as determined by the membrane marker enzymes, alkaline phosphatase (MVM) and adenylate cyclase (BM). There was minimal cross-contamination of the two isolated plasma membrane fractions and the yields obtained were 26% (MVM) and 21% (BM) compared to the initial homogenate. The MVM and BM fractions were free from contamination by mitochondrial or lysosomal membranes and showed only minor contamination by microsomal membranes. The two membrane fractions were also tested for the presence of non-syncytial plasma membranes by electrophoretic immunoblotting. Contamination of both MVM and BM by fibroblast, endothelial, macrophage and cytotrophoblast plasma membranes amounted to less than 15% of the total membrane protein as determined by immunoblotting. Vesicle orientation, determined from the latency of specific concanavalin A binding, was 88 ± 4% right-side out for MVM and 73 ± 12% right-side out for BM. This simple preparative procedure produces a high yield of both MVM and BM from human placenta. The analytical data demonstrates that 'paired' MVM and BM fractions derived from the same placental tissue have a high purity in terms not only of contamination by intracellular membranes, but also in terms of contamination by non-syncytial plasma membranes.

Introduction

The human placental syncytiotrophoblast is a polarized epithelial structure which forms the main barrier between mother and fetus. This epithelium is composed of large, multinucleate cells which result from the fusion of the precursor cytotrophoblast cells, producing a syncytium. The syncytial architecture precludes the preparation of intact syncytiotrophoblast cells while the polarized structure necessitates the use of both the fetal-facing basal membrane (BM) and the maternal-facing microvillous membrane (MVM) in any analysis of transplacental transport. Development of methods for the isolation and purification of paired MVM and BM has provided avenues for the investigation of the structure and function of specific placental transport mechanisms.

The most common preparations of MVM employ two key steps. The first is an isotonic agitation step [1] which causes shearing of microvilli from the microvillous surface, separating them from the rest of the placental structure. The second is a Mg^{2+}-precipitation step, in which non-microvillous membranes are aggregated by Mg^{2+} and can be separated from the MVM by low-speed centrifugation [2,3]. BM have been prepared by three methods; Boyd et al. [4] used homogenization and zonal centrifugation techniques to prepare a fraction enriched in Na^+/K^+-ATPase and adenylate cyclase, both of which are located on the basal but not microvillous surface. Kelley et al. [5] used repeated sonication, hypotonic lysis and density gradient centri-

Abbreviations: MVM, microvillous membrane(s); BM, basal membrane(s).

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fugation to isolate a membrane fraction enriched in β-adrenergic and ouabain binding. Brunette et al. [6] used a homogenization, Mg^{2+} precipitation and discontinuous Ficoll-gradient centrifugation to prepare both MVM and BM which were enriched in alkaline phosphatase and β-adrenergic binding.

A common feature of most MVM and BM preparations is the low yield of the purified membrane fractions, as measured by membrane protein and membrane marker enzyme recovery. This raises the possibility that these membrane preparations may be selected subfractions of the *in vivo* membrane surface. Thus the transport properties determined using these fractions may differ significantly from those of the *in vivo* surface. An additional problem is that contamination of these preparations by non-syncytial plasma membranes, such as those from placental fibroblasts and endothelial cells, has not generally been determined [1,2,4–9].

In developing a membrane preparation, an important consideration is the applications for which the preparation is intended in the case of placental membrane preparations, most studies are concerned either with transport of essential nutrients across both microvillous and basal membranes or with the regulation and gestational development of transport processes. Associated with these is a growing interest in the role of the placenta in various pathologic processes of pregnancy, such as intrauterine growth retardation and premature birth. To examine these aspects of placental function, investigation of events at both microvillous and basal surfaces is often necessary. Development of a 'paired' preparation in which purified MVM and BM are isolated from a piece of the same placental tissue, is therefore desirable, especially when limited quantities of tissue may be available. A paired placental membrane preparation is also of importance when the genetic and clinical heterogeneity of human tissue samples is considered.

Methods and Materials

Vesicle preparation

Term placentae (38–41 weeks) from uncomplicated pregnancies were obtained after normal vaginal delivery and prepared within 20 min. All procedures were performed at 4°C unless otherwise stated. After removal of the cord, amniochorion, chorionic plate and a decidual layer (0.25 cm thick), the tissue was cut into 1 × 1 cm pieces and washed with 0.9% NaCl to remove blood. The tissue was homogenized in a Waring blender for 2 min in 250 mM sucrose, 10 mM Tris-Hepes (pH 7.0), containing 5 mM EGTA, 5 mM EDTA and 1 mM PMSF, added to inhibit proteinase action (buffer 1; 25% w/v). The homogenate (H) was centrifuged at 10 000 × *g* for 15 min and the supernatant was removed and retained. The low-speed pellet was resuspended in buffer 1 (25% w/v) and rehomogenized for 2 min. The second homogenate was centrifuged at 10 000 × *g* for 15 min and the supernatants from both centrifugations were combined and filtered through gauze. The combined supernatant was centrifuged at 47 500 × *g* for 60 min and the resulting pellet (P2) was resuspended in one volume of buffer 1 (approx. 15 mg membrane protein/ml).

To separate MVM from the other membrane particulates, solid $MgCl_2$ was added to the P2 suspension (final concentration 12 mM) and the mixture was stirred on ice for 20 min. This mixture was then centrifuged at 2500 × *g* for 15 min to sediment the Mg^{2+}-aggregated fraction. The supernatant containing the MVM was centrifuged at 47 500 × *g* for 30 min and the resulting pellet was washed in 250 mM sucrose, 10 mM Tris-Hepes (pH 7.0) (buffer 2). The final MVM pellet was resuspended in buffer 2 to a concentration of approx. 15 mg/ml, frozen in liquid N_2 and stored at −70°C.

The Mg^{2+}-aggregated pellet (containing the BM) was resuspended in buffer 2 immediately after low-speed sedimentation, homogenized thoroughly in a Dounce homogenizer and layered on to a sucrose step gradient. The step gradient was constructed in a 40 ml Beckman SW 28 centrifuge tube using a bottom fraction with density 1.190 g/cm^3 (6 ml) and a middle fraction with density 1.165 g/cm^3 (17 ml) on to which was layered 15 ml of the resuspended Mg^{2+}-aggregated fraction. All the steps contained 10 mM Tris-Hepes (pH 7.0). The gradient was centrifuged for 60 min at 141 000 × *g* using a Beckman SW 28 swing-out rotor (Beckman Instruments, Palo Alto, CA) with slow acceleration and deceleration through 140 rpm. The fraction which collected at the interface between the bottom and middle steps was removed, diluted with ten volumes of buffer 2, homogenized in a Dounce homogenizer and centrifuged at 47 500 × *g* for 30 min. The resulting BM pellet was resuspended in buffer 2, washed, frozen in liquid N_2 and stored at −70°C. For analytical purposes, the Mg^{2+}-aggregated suspension was initially fractionated on a continuous sucrose gradient. This was made up as a 32–44% (w/w) sucrose gradient containing 10 mM Tris-Hepes (pH 7.0) and was centrifuged for 16 h at 141 000 × *g*.

Assays

Alkaline phosphatase, cytochrome *c* oxidase, NADPH–cytochrome-*c* reductase and Na^+/K^+-ATPase activities were assayed using previously published methods [10]. Adenylate cyclase activity was assayed by the method of Saloman et al. [11], in the presence of 10^{-5} M forskolin, 0.4 mM ATP and 4 mM $MgCl_2$. Freezing samples promptly in liquid N_2 and storage at −70°C was necessary to preserve adenyl cyclase activity. *N*-Acetyl-D-glucosaminide deacetylase was assayed by a modification of the method of Bag-

giolini et al. [12]; after incubation with substrate (*p*-nitrophenyl *N*-acetyl-D-glucosaminide) for 30 min at room temperature, the reaction was stopped by addition of trichloroacetic acid (7.5% w/v, final concentration) and the samples were centrifuged to separate denatured material. The acid supernatant was neutralized by addition of 1 M NaOH and the absorbance at 405 nm was measured. Protein was assayed by the method of Bradford [13].

Orientation assays for vesicle fractions were carried out by measurements of the binding of concanavalin A-fluorescein isothiocyanate (Con A-FITC) before and after vesicle lysis. Vesicle samples (approx. 200 μg membrane protein) were incubated with Con A-FITC (10 μg/ml) in 140 mM KCl, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 10 mM Tris-Hepes (pH 7.2) for 10 min at room temperature. After precipitation of the vesicles by high-speed centrifugation, Con A-FITC fluorescence in the supernatant was measured in an SLM 8000C spectrofluorimeter (SLM Instruments, Urbana, IL) using a 515 nm emission cut-on filter (excitation 490 nm). Binding was measured in the presence and absence of 0.5 M methyl α-D-mannopyranoside (which displaces specifically bound concanavalin A) and the difference between the two measurements was used as a measure of the specific binding of Con A-FITC. The fraction of right-side out vesicles was taken as the ratio of specifically bound Con A-FITC fluorescence measured before and after lysis, which was performed by a liquid N_2, freeze-thaw cycle repeated three times.

Immunoblotting

H, MVM and BM samples were tested for the presence of a number of protein markers by immunoblotting. Samples were solubilized by sonication in 2% SDS and single-dimension SDS-PAGE was carried out using 50–100 μg of membrane protein by the method of Laemmli [14], on 5 × 10 cm 10% gels (0.75 mm thick). Protein was electrophoretically transferred to nitrocellulose sheets (16 h, 50 mA/gel) which were blocked with casein and probed with primary antibody (5–10 μg antibody/blot) for 3 h. Blots were washed and probed for 1 h with the appropriate ^{125}I-labelled IgG as a secondary probe, followed by autoradiography. The intensity of the labelled bands was determined by densitometry.

A series of membrane preparations from isolated cells were used as positive controls for possible contaminating plasma membranes. Human placental fibroblasts were obtained from Dr. Susan J. Fisher, prepared by the method of Fisher et al. [15]. Placental cytotrophoblast and macrophages were prepared by the method of Kliman et al. [16] by Dr. Elliot K. Main. Cultured human umbilical vein endothelial cells prepared by the method of Rodgers et al. [17] were obtained from Dr. Robert N. Taylor. In all cases, cells were sonicated in 10 mM Tris-Hepes (pH 7.0) and the high-speed pellet obtained after sonication was solubilized as described above for use in electrophoresis.

Chemicals

[α-^{32}P]ATP was obtained from New England Nuclear (Boston, MA). Nitrocellulose was obtained from Schlier and Schuell (Keene, NH) and chemicals for electrophoresis from Bio-Rad (Richmond, CA). Concanavalin A-fluorescein isothiocyanate was purchased from Molecular Probes (Eugene, OR). Anti-vimentin (clone v9) and anti-cytokeratin 18 (clone CK-2) were obtained from Boehringer Mannheim ((Indianapolis, IN). An antibody specific to the A3 region of the HLA heavy chain (Q1/28) was a gift from Drs. V. Quaranta and N. Cohen. All other chemicals were obtained from Sigma Chemical Company (St Louis, MO) or Fisher Scientific (Fair Lawn, NJ).

Results

Preparative steps

A number of conditions were tested to determine the optimal preparative procedure. The maximum centrifugation speed which could be used to remove cellular debris and intracellular particles after homogenization, without loss of the released microvillous and basal membranes, was determined. Homogenized samples from three separate placenta preparations were centrifuged at 4000, 6000, 8000, 10000 and 12000 × *g* for 15 min. Assay of the supernatants for MVM and BM enzymatic markers (alkaline phosphatase and Na^+/K^+-ATPase) revealed that there was no significant loss of either marker using forces up to and including 10000 × *g*, but over 10000 × *g* significant losses were observed, especially in the basal Na^+/K^+-ATPase (data not shown). The initial centrifugation step alter homogenization was therefore carried out at 10000 × *g* for 15 min.

The minimum degree of tissue homogenization necessary to release MVM and BM was then measured. Washed tissue samples were homogenized in the same volume of buffer 1 for 30, 90, 120 or 240 s. The homogenized samples were centrifuged at 10000 × *g* for 15 min and the resulting supernatant samples were assayed for alkaline phosphatase and Na^+/K^+-ATPase (Fig. 1). While release of the MVM marker (alkaline phosphatase) was augmented 27% by a 8-fold increase in the homogenization time, release of the basal membrane marker (Na^+/K^+-ATPase) was doubled. A homogenization time of 120 s was chosen to maximize release of membrane while minimizing sample warming during homogenization.

Addition of a second homogenization step to increase release of MVM and BM was tested. For five separate preparations, tissue was homogenized for 120

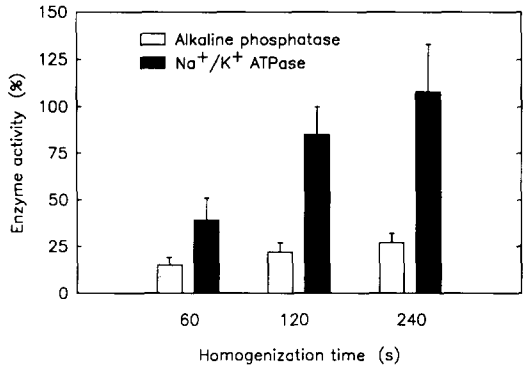

Fig. 1. Effects of homogenization time. The graph shows the percent increase in the release of microvillous and basal membranes after homogenization for various periods of time, compared to homogenization for 30 s. Tissue was homogenized for 30, 60, 120 and 240 s then centrifuged at low speed (2500 × *g*; 15 min). The supernatants from these samples were tested for the presence of alkaline phosphatase (microvillous membrane; open bars) and Na^+/K^+-ATPase (basal membrane; filled bars) and the results for 60, 120 and 240 s ($n = 5$) are plotted as a percentage of the values obtained for the 30 s supernatant.

s, centrifuged at 10000 × *g* for 15 min, the supernatant removed (and retained) then the pellet was resuspended in buffer 1 and rehomogenized under the same conditions. The second homogenate was centrifuged at 10000 × *g* for 15 min and the supernatant collected. Samples from the first homogenate and the first and second supernatants were assayed for alkaline phosphatase and Na^+/K^+-ATPase activities. Taking the first homogenate sample activity as 100%, the alkaline phosphatase and Na^+/K^+-ATPase activities in the first supernatant were 81 ± 7% and 49 ± 9%, while the second supernatant contained a further 10 ± 3% and 34 ± 5%, respectively. These results demonstrate that although most of the MVM marker was released into the supernatant by the first homogenization step, a significant quantity (34%) of the BM marker was released by the second homogenization. A second homogenization step was therefore included in the final procedure.

The degree to which Mg^{2+}-precipitation of the BM-containing fraction was reversible was tested by comparing vesicles resuspended in buffer 2 to resuspension in the presence of Na^+, EDTA and albumin. Resuspension in the presence of Na^+ was performed to determine whether the Mg^{2+} responsible for aggregation could be displaced by Na^+, permitting a greater degree of resuspension. Resuspension in EGTA was carried out in an attempt to chelate Mg^{2+}, while albumin was added because of its anti-aggregatory properties. Membranes pelleted by the low-speed centrifugation after Mg^{2+} aggregation were homogenized using a Dounce homogenizer in a small volume of buffer 2 then divided into four aliquots and diluted with (i) buffer 2 alone or buffer 2 plus (ii) 100 mM NaCl, (iii) 100 mM NaCl, 10 mM EGTA or (iv) 100 mM NaCl, 10 mM EGTA, 1 mg/ml bovine serum albumin. The pelleted membranes were rehomogenized in the Dounce homogenizer and centrifuged at 2500 × *g* for 15 min. The supernatant and pellet were assayed for Na^+/K^+-ATPase activity. Of the combined total activity in the homogenized Mg^{2+} precipitate (100%), (i) resuspension in buffer 2 alone produced 79 ± 1% in the supernatant, (ii) resuspension in NaCl left 60 ± 14% in the supernatant, (iii) NaCl/EGTA produced 57 ± 24% and (iv) NaCl/EGTA/albumin produced 54 ± 22% ($n = 3$). Buffer 2 was used without additions for resuspension in the final procedure. In several experiments, the Mg^{2+} precipitate was frozen and stored prior to homogenization, dilution and centrifugation. In these instances, the majority of Na^+/K^+-ATPase activity (> 75%) was pelleted by centrifugation at 2500 × *g* for 15 min and could not be resuspended in any medium.

The final conditions to be examined were the sucrose gradient steps. To establish the density of the BM fraction, the Mg^{2+}-precipitated pellets from six separate placental preparations were centrifuged on continuous sucrose gradients (32–44% (w/w)) for 16 h at 141000 × *g*. The gradients were fractionated and the densities, 280 nm absorbance (protein content) and Na^+/K^+-ATPase activity were measured for each fraction. Fig. 2 shows a typical gradient profile. One major peak of Na^+/K^+-ATPase activity is apparent at a peak density of approx. 1.175 g/cm³. Over six preparations similar results were obtained with a mean peak density for the Na^+/K^+-ATPase activity at 1.173 ± 0.005 g/cm³. Sucrose density steps of 1.165 g/cm³ and 1.190 g/cm³ were chosen to bracket the peak Na^+/K^+-ATPase (BM) density for the final preparative procedure.

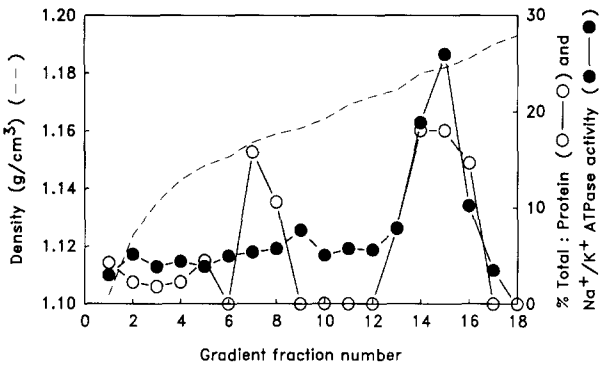

Fig. 2. Continuous sucrose gradient of Mg^{2+}-aggregated membranes. Mg^{2+}-aggregated material was resuspended in buffer 2 and layered on to a sucrose gradient (32–44% w/w) containing 10 mM Tris-Hepes (pH 7.0). The samples were centrifuged in a swing-out rotor for 16 h at 141000 × *g* and fractionated. The fractions were assayed for Na^+/K^+-ATPase activity (filled circles), and the density (g/cm³; dashed line) and protein content (A_{280}; open circles) were measured for each sample. These parameters are plotted against gradient position (fraction number) going from the top of the gradient (No. 1) to the bottom (No. 20), for a typical example.

TABLE I

Activities and enrichment factors for alkaline phosphatase and adenylate cyclase

Fraction	Alkaline phosphatase		Adenylate cyclase	
	spec. act. [a]	Enrich-ment	spec. act. [c]	Enrich-ment
H	105 ± 74	1.0	Not done	–
P2	297 ± 255	2.8	15.3 ± 2.6	1.0
MVM	2069 ± 807	19.7 [b]	1.2 ± 0.4	0.1
BM	163 ± 108	1.5	161.0 ± 29.0	10.5 [d]

[a] nmol/min per mg membrane protein.
[b] MVM/H.
[c] pmol/min per mg membrane protein.
[d] BM/P2.

Activities and enrichment of microvillous and basal membranes

MVM enrichment and yield were assessed using the enzyme marker alkaline phosphatase. BM enrichment and yield were measured using forskolin-stimulated adenylate cyclase as a marker. Activity of the latter enzyme is enhanced by a variety of soluble (cytosolic) factors and therefore the enrichment and yield in the BM were calculated by comparison with the high-speed membrane pellet (P2), rather than the homogenate (H). Table I shows the specific activities and enrichment factors for alkaline phosphatase and adenylate cyclase in the H, P2, MVM and BM fractions. These results demonstrate that there is substantial enrichment of MVM (20-fold) and BM (11-fold) by this procedure. The cross contamination of the BM fraction with MVM was low, since alkaline phosphatase in the BM fraction showed only a 1.5-fold enrichment compared to homogenate. The MVM fraction was essentially free of basal membrane since the adenylate cyclase activity in the MVM fraction was reduced to less than one tenth of the activity observed in the P2 fraction.

The yield of protein, MVM and BM markers is shown in Table II for H, P2, MVM and BM fractions. The recovery data calculated from alkaline phosphatase and adenylate cyclase activities indicates that this preparation produced a yield of 26% for MVM and 25% for the BM. It should be noted that the recovery figure for the BM is relative to the P2 fraction rather than homogenate. The membrane protein recoveries for MVM and BM were 0.66 ± 0.20 mg/g and 0.84 ± 0.5 mg/g wet weight of placenta ($n = 5$).

Contamination of microvillous and basal membranes by intracellular membranes

The contamination of MVM and BM fractions by intracellular membranes was assessed by measuring the activities of enzymes which are specific markers for these membranes. Mitochondrial contamination was measured using cytochrome-*c* oxidase. Contamination

TABLE II

Recovery of protein, alkaline phosphatase and adenylate cyclase

Fraction	Protein (mg/g wet wt)	Alkaline phosphatase (%)	Adenylate cyclase (%)
H	76.9 ± 12.1	100	
P2	4.3 ± 1.0	42 ± 22	100
MVM	0.66 ± 0.20	26 ± 15	0.2 ± 0.1
BM	0.84 ± 0.48	3 ± 2	25.1 ± 7.2

by microsomal membranes was determined from NADPH–cytochrome-*c* reductase activity and lysosomal contamination was measured using *N*-acetylglucosaminide deacetylase. The results of these assays are shown in Table III. These data show that MVM and BM were not contaminated by either mitochondrial or lysosomal membranes and that there was only a small degree of contamination by microsomal membranes.

Contamination of microvillous and basal membrane fractions by non-syncytial plasma membranes

It is probable that the homogenization method used here to release MVM and BM also disrupted non-syncytial cells in the placental tissue. It was necessary therefore to determine the degree of contamination by the plasma membranes from these cells in the syncytiotrophoblast membrane fractions. Morphometric data suggests that for term placentae, the most prevalent non-syncytial cell types are placental fibroblasts and capillary endothelium [18], however cytotrophoblast and macrophage contamination was also considered.

Analysis of the contamination of MVM and BM fractions was performed by immunoblotting, using a variety of probes to contaminant cell antigens. The choice of probes for fibroblast and endothelial cell plasma membranes (vimentin) and for syncytiotrophoblast membranes (cytokeratin) was based on previous literature observations [19–22]. The standard probes for

TABLE III

Intracellular membrane marker enzyme activity in microvillous and basal membrane fractions

Enzyme	Enzyme activity (nmol/min per mg); (enrichment)			
Fraction:	H	P2	MVM	BM
Cytochrome-*c* oxidase	8.0 ± 3.1 (1.0)	11.3 ± 0.6 (1.4)	8.3 ± 2.1 (1.0)	not detectable
NADPH–cytochrome-*c* reductase	4 ± 1 (1.0)	18 ± 3 (4.5)	9 ± 2 (2.7)	12.0 ± 7 (3.0)
N-Acetyl-glucosaminide deacetylase	7.3 ± 2.8 (1.0)	13.9 ± 4.3 (1.9)	7.3 ± 2.1 (1.0)	2.8 ± 1.6 (0.4)

TABLE IV

Cross reactivity between antibodies and placental cell types

	Vimentin	Q1/28	Cytokeratin
Fibroblasts	+ +	+ +	–
Endothelium	+ + +	+ +	–
Macrophages	+	+ + +	–
Syncytiotrophoblast	–	–	+ + +

macrophages are antibodies to the HLA-A, B, C or DR antigens, however antibodies to HLA-A, B and C are not available for immunoblotting purposes and it has been suggested that fetal macrophages may not demonstrate HLA-DR. We therefore used an antibody prepared against the A3 region of the HLA heavy chain (Q1/28; Quaranta, V. and Cohen, N., unpublished data). A series of positive immunoblotting controls were carried out using the membrane particulate fraction of cells identified as possible contaminants. The cell types which were identified by the various antibodies and the degree of response are shown in Table IV.

The three antibodies (antivimentin, anticytokeratin, Q1/28) were tested in immunoblotting experiments against H, MVM and BM fractions obtained from four placental preparations and the data obtained was compared to results from the positive controls (reference contaminant cell samples). Quantitative results for the three antibodies (anti-vimentin, Q1/28, anti-cytokeratin) for four separate placental preparations are shown in Table V, in arbitrary density units per mg protein. Compared to the fibroblast, endothelial and homogenate samples, MVM and BM samples showed little or no staining for vimentin. Blots probed with Q1/28 showed staining in H, MVM and BM samples which was weak compared to the strong responses observed for macrophages, fibroblasts and endothelial cell samples. Cytokeratin staining was strong in H, MVM and BM samples. The vimentin data demonstrates that the contamination of the MVM and BM fractions by fibroblasts and endothelial plasma membranes was negligible. The quantity of vimentin-containing membranes in the MVM fraction was reduced by 80% relative to homogenate and by 66% in the BM compared to homogenate. In contrast, the MVM and BM fractions showed an enrichment in cytokeratin compared to homogenate, demonstrating purification of the syncytiotrophoblast membranes. The results using Q1/28 show that the quantities in all the experimental fractions are small relative to the reference macrophage sample. Thus while there is no significant difference in the quantity of antigen per mg protein between homogenate, MVM and BM fractions, the degree of contamination by Q1/28 positive membranes is less than 10%. Overall, the contamination by endothelial cells, fibroblasts and macrophages is less than 10% as measured by Q1/28, the antibody which is an identifier for all three contaminant cell types.

TABLE V

Non-syncytial antigen content of microvillous and basal membranes

Antibody	Control (=1.0)	Antigen density compared to control [a]		
		H	MVM	BM
Anti-vimentin	Endothelial cell	0.59 ± 0.14	0.12 ± 0.01	0.20 ± 0.05
Q1/28	Macrophage	0.05 ± 0.01	0.08 ± 0.02	0.09 ± 0.04
Anti-cytokeratin		1.0	1.14 ± 0.33	1.47 ± 0.38

[a] Anti-vimentin and Q1/28 values normalized to control values of 1.0. Anti-cytokeratin values for MVM and BM normalized to a homogenate value of 1.0.

Contamination of MVM and BM fractions by cytotrophoblast was tested by tracer experiments. Cytotrophoblast cells prepared from term human placenta were covalently labelled with ^{125}I by the Iodogen method (Pierce, Rockford, IL) using approx. 4 μg of Iodogen reagent for 10^7 cells. The labelled cells, separated from free label by centrifugation and washing, were used to trace cytotrophoblast membranes in the paired membrane preparation. Labelled cells were added to the washed placental tissue immediately prior to homogenization (approx. $50 \cdot 10^6$ cpm/50 g tissue). Samples were taken throughout the preparation to determine the fate of the labelled cytotrophoblast plasma membranes. The labelled cells were disrupted by homogenization since > 85% of the label remained in the supernatant after the $10000 \times g$ centrifugation step. At the end of the isolation procedure, the final MVM and BM fractions contained less than 2% of the label ($n = 3$).

Orientation of microvillous and basal membrane vesicles

Microvillous vesicles prepared by the agitation method are oriented with the cytoplasmic face towards inside of the vesicle, i.e., right-side out vesicles [9,23]. Basal membrane vesicles from other tissues have been shown to have a mixed orientation with approx. 60% right-side-out and approx. 40% inside-out vesicles. The vesicle orientation of the MVM and BM fractions was determined by measuring the quantity of specifically-bound concanavalin A-fluorescein isothiocyanate (Con A-FITC) before and after lysis of the vesicles as described in Methods. The ratio of specifically bound Con A-FITC determined before and after lysis is a measure of the fraction of right-side-out vesicles. The ratio for the MVM was 0.88 ± 0.04, while the ratio for BM was 0.73 ± 0.12 ($n = 5$).

Discussion

In establishing a model of resolved basal and microvillous membranes to study placental transport, there are conflicting goals. The first is to isolate membrane

fractions of the highest possible purity, eliminating contamination by intracellular organelles and other plasma membranes. However most biological membranes are heterogenous, containing a variety of differing domains and intensive purification techniques may create subpopulations which are not representative of the entire membrane. There is evidence for membrane heterogeneity in human placental microvillous membranes; lipid domain separation [24], non-uniform distribution of insulin receptors [25] and the subfractionation of microvillous vesicles [8,10]. The second goal is to obtain a membrane fraction which is representative of the *in vivo* surface, despite the heterogeneity. There is a balance to be drawn between membrane purification and the isolation of a fraction which is representative of the *in vivo* surface. The procedures outlined in this report are an attempt to strike that balance and obtain purified but representative fractions of placental microvillous and basal membranes. A combination/modification of current techniques (agitation/sonication) was considered as a possible pattern for simultaneous MVM and BM preparation, however the low yields obtained using these methods and thus the questions of subfractionation and the representative nature of the membrane fractions mitigated against this route. The homogenization procedure was deemed necessary to produce a high yield of representative membranes, despite the problems of contamination thus created.

In the analysis of transplacental transport or signalling processes, it may be necessary to investigate events at both the microvillous and basal membrane surfaces. For a number of reasons it is desirable to examine membrane functions in microvillous and basal membranes from the same tissue. One reason is to be able to make quantitative determinations of the coupling between microvillous and basal processes. For example, knowledge of both microvillous and basal surface areas and permeabilities are required to quantitate transplacental transport. Another reason for requiring membranes from the same tissue is for exploration of placental pathophysiologies where alteration in placental function is suspected but the site of the lesion(s) is unknown. In cases where tissue is obtained from preterm or pathological placentae, the quantity of tissue available may be limited, necessitating isolation of both membranes from the same tissue sample. Finally, the clinical and genetic heterogeneity observed in human placental tissue, which makes inter-tissue comparisons difficult, provides another justification for preparing microvillous and basal membranes from the same tissue sample.

The preparation of 'paired' microvillous and basal membranes from the same tissue sample has been attempted previously by Whitsett et al. [26]. Their method used the agitation technique [1] to produce a microvillous membrane fraction, after which the remaining tissue was used to prepare a basal membrane fraction by homogenization and differential centrifugation. Although an enriched microvillous fraction was produced, a clearly identifiable basal fraction was not evident. In addition, both fractions suffered from cross-contamination and contamination by intracellular membranes. More recently, preparation of basal and microvillous membranes has been described by Brunette et al. [6] using a method somewhat similar to that described here. Although there was marked enrichment of microvillous (32-fold) and basal (24-fold) fractions, substantial cross contamination reduced the enrichment of microvillous membranes to approx. 6-fold relative to basal membranes and the enrichment of basal membranes to approx. 4-fold relative to microvillous membranes. A new MVM preparation has been described recently which uses homogenization rather than agitation [9]. While this preparation increased the yield of microvillous membranes, there was significant enrichment of basal membranes in the same fraction. Contamination by intracellular membranes and non-syncytial plasma membranes was not determined for either of the preparations employing homogenization. This report is the first to describe the simultaneous separation and purification of both microvillous and basal membranes with high enrichment and minimal contamination.

The enrichment of the MVM in this preparation was 20-fold as judged by alkaline phosphatase activity, while the recovery was 26%. These values are similar or higher than the enrichments and recoveries reported by Booth et al. ([3]; 24-fold, 5%), Truman et al. ([8]; 20-fold, 5%) and Glazier et al. ([9]; 18-fold, 16%). The enrichment of the BM (11-fold) is similar to that found by Boyd et al. ([4]; 7-fold), although lower than the 45-fold enrichment of dihydroalprenolol binding observed by Kelly et al. [5]. The enrichment of the BM is likely to be higher than 11-fold if comparison is made to the homogenate rather than the P2 fractions. Applying the nominal P2/H enrichment factor for alkaline phosphatase observed in MVM to the enrichment figures for adenyl cyclase gives a BM/H enrichment of 16-fold for adenyl cyclase. The adenyl cyclase recovery in the BM fraction is also compared to that in the P2 fraction rather than the homogenate and the real recovery will be lower than 25%. The extraction figures determined in the experiments to investigate double homogenization showed a Na^+/K^+-ATPase (BM) recovery of 83% in the low-speed supernatant after homogenization and centrifugation; applied to the adenylate cyclase recovery figures, this produces a final recovery of 21%. This is substantially higher than that observed by Boyd et al. [4] or by Kelley et al. [5] (less than 8%). It is likely that the basal membrane markers used here (adenyl cyclase) and elsewhere (β-adrenergic binding, Na^+/K^+-ATPase) to measure membrane yield are not exclusive to the basal membrane but may also be found on the plasma membranes of other placental cells. In combination with the

immunoblotting/tracer results showing minimal contamination by non-syncytial plasma membranes, the adenyl cyclase results can be used with greater confidence.

The yield of membrane protein per g wet weight of placental tissue (0.66 mg/g, MVM; 0.84 mg/g, BM) is considerably higher than previous published reports (0.09–0.13 mg membrane protein/g wet weight of placenta, MVM; 0.06 mg/g, BM; [3,5,8]), with the exception of the homogenization method described by Glazier et al. for MVM ([9]; 0.6 mg/g).

The high yield from this preparation was not produced at the expense of membrane purity. Cross-contamination of the microvillous and basal fractions, was low, as evidenced by the decreased enrichment of adenylate cyclase in the microvillous membrane fraction and the lack of alkaline phosphatase enrichment in the basal fraction. BM showed a small degree of contamination by microsomal membranes, as determined by NADPH–cytochrome-*c* reductase activity, similar to that found in the basal preparation of Kelley et al. [5]. Otherwise, contamination of these two membrane fractions by intracellular membranes was minimal.

Immunoblotting provides a quantitative assessment of the contamination of the MVM and BM fractions by non-syncytial plasma membranes. Overall, the fraction of membrane protein which can be attributed to contaminant fibroblast, endothelial, macrophage and cytotrophoblast membranes is less than 12% for both MVM and BM. The quantitation of contaminants is based on the assumption that the density of antigen per unit of membrane protein is the same in the reference contaminant cell samples as that in the actual contaminant membranes in the MVM and BM fractions. This appears to be a reasonable assumption since the fibroblast, cytotrophoblast and macrophage cell samples are obtained directly from placental tissue. Although the endothelial cells are not obtained directly from the placenta, their source, the umbilical vein, is likely to contain endothelial cells similar to those in the placental capillaries. A separate error in quantitation may arise from the nature of the reference contaminant cell preparations. Reference contaminant cell membrane fractions were obtained from the pellet of a high-speed centrifugation after lysis of the contaminant cells. As such, these fractions contain intracellular membranes (hence membrane protein) in addition to the plasma membranes but no antigen associated with the intracellular membrane protein. The antigen densities of the reference contaminant cell plasma membranes obtained from the immunoblots (arbitrary density units/mg membrane protein; Table V) are therefore likely to be lower than values for pure contaminant plasma membranes, due to an overestimation of the plasma membrane protein content (by inclusion of the intracellular protein). Thus the contamination figures are, in all probability, smaller than those shown in Table V and the overall contamination less than the 12% determined here. We judge the quantity of non-syncytial plasma membrane contamination to be acceptably low enough for measurements of transport and signal transduction. Further purification to reduce contamination by intracellular or non-syncytial contaminants would be likely to reduce drastically the yield of this preparation and lead to questions regarding subfractionation. Because of the generalized methods employed, it is likely that this preparation will also be suitable for obtaining syncytiotrophoblast membranes from pre-term and other pathological tissues.

In summary, this report describes a preparation which is capable of producing a high yield of paired syncytiotrophoblast microvillous and basal membranes from the same tissue. The isolated fractions display minimal cross-contamination, minimal contamination by intracellular membranes and low levels of contamination by non-syncytial plasma membranes. This preparation will be suitable for a variety of applications including measurements of membrane transport and signal transduction.

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