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A biochemical-morphological study on microvillus plasma membrane development

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Abstract

The microvillus plasma membrane of the human placental syncytiotrophoblast at term has been extensively studied, while little is known about the characteristics of its development. The aim of the present work was to compare functional and structural properties of this membrane at early and term gestational age. Ten normal term placentas (40 weeks) and ten placentas at 10 weeks of gestational age were studied. The Na^+/K^+-ATPase activity is significantly decreased in the syncytiotrophoblast plasma membrane obtained from term placentas as compared to the early ones, with significant variation of maximum velocity (V_{max}). The microviscosity, evaluated by the P parameter of DPH and S_n parameters of 5- and 16-NS, is increased in the term placentas compared to the early placentas. This alteration is accompanied by an increased cholesterol to phospholipids ratio in term placentas, while there is a decreased unsaturated to saturated fatty acid ratio. As follows from morphological studies, an increased mean diameter in the E face was observed in the term placenta with respect to the early placenta. The distribution factor DF, wich indicates the particle aggregation state, decreased in the E face in the term placenta as compared to the early one. The present biochemical morphological study shows that a deep modification of the membrane is at the basis of the syncytiotrophoblast plasma membrane development.

Key words: Microvillus plasma membrane; Human placenta; Development; ATPase, Na^+/K^+-; Fluidity; Freeze-fracture

1. Introduction

Human syncytial trophoblast is known to play fundamental roles in pregnancy [1–5]. Indeed, the placenta acts as a fetal lung, kidney and intestine and, therefore, placental Na^+ and K^+ transport is of great importance for fetal growth and development. Furthermore, the transport of several essential nutrients (phosphates, amino acids) is coupled to the transmembrane sodium gradient across the plasma membrane of the placental syncytiotrophoblast.

The microvillus plasma membrane of the human placental syncytiotrophoblast is in direct contact with maternal blood, so it mediates the transport of nutrients and immunoglobulins from maternal to fetal circulation; it also functions as an endocrine organ, secreting steroids and proteic hormones [6]. Biochemical and morphological studies [7–8] have been performed on the plasma membrane obtained from normal term placentas, while little is known about the characteristics of early gestational ages [9].

To add new insight to this open question an ultrastructural and biochemical study was performed.

2. Materials and methods

Ten normal term placentas (40 weeks) and ten placentas at 10 weeks of gestational age were studied. The microvillus plasma membranes were obtained according to the method of Whitsett and Wallick, with minor

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modifications as previously reported [10–11]. The placental tissue was cut into fragments and washed briefly in Krebs-Henseleit buffer. About 300 g of tissue was then placed in ice-cold NaCl 150 mmol l^{-1} and stirred gently for 30 min. After gauze-filtration, the filtrate was centrifuged at 20000 × *g* in a Kontron Centrikon (Germany) H-401 centrifuge, using an A824 rotor. The supernatant obtained was centrifuged at 100000 × *g* in a Kontron Centrikon T-2070 centrifuge (using a TFT 80.13 rotor), resuspended and then centrifuged at 7000 × *g* for 10 min. Biochemical markers were tested as previously reported [7,8]. Protein concentration was assayed according to the method of Lowry et al. [12].

2.1. Na^+/K^+-ATPase assay

The membrane Na^+/K^+-activated Mg^{2+}-dependent ATPase activity was determined using the Kitao method [13], with minor modifications as previously reported [14,15]. The ATPase activity was assayed by incubating the membranes at 37°C in a medium containing $MgCl_2$(5 mmol l^{-1}), NaCl (140 mmol l^{-1}), KCl (14 mmol l^{-1}) in Tris-HCl 40 mmol l^{-1} (pH 7.70) at 37°C for 10 min.

The ATPase reaction was started by the addition of 3 mmol l^{-1} Na_2ATP and then stopped by the addition of 1 ml of 15% trichloroacetic acid. Inorganic phosphate (P_i) hydrolyzed from reaction was then measured according to the Fiske and SubbaRow method [16]. Enzyme activity was expressed as the difference in inorganic phosphate released in the presence and absence of 10 mmol l^{-1} ouabain, respectively. The ATPase activity assayed in the presence of ouabain was subtracted from the total Mg^{2+}-dependent ATPase activity to calculate the activity of the ouabain-sensitive Na^+/K^+-ATPase. The results are expressed as μmol P_i/mg membrane protein per h. In order to study the kinetics of the enzyme, we assayed the Na^+/K^+-ATPase activity in microvillous plasma membranes obtained from each subject of the study group using increasing concentrations of the substrate Na_2ATP (2.5–20 mM). From these data we calculated the Michaelis-Menten constant (K_m) and the maximum velocity (V_{max}) of the enzyme in syncytiotrophoblast plasma membranes from both early and term placentas [17].

2.2. Membrane cholesterol and phospholipid content

Lipids were extracted from the membranes according to the method of Folch [18] by using 10 ml chloroform/methanol (2:1, v/v) per ml of membrane suspension. The cholesterol concentration was then determined by the method of Zak [19] and the total phospholipids according to Bartlett [20].

2.3. Fatty acids

Fatty acids were identified according to Wing et al. [21], using a Carlo Erba Fractovap 2000 gas chromatograph equipped with a flame ionization detector, the glass column SP-2330 GP 10% on Chromosorb WAW 100–120 mesh, 2 m × 2 mm i.d., column temperature was 190°C, injector temperature was 210°C, detector temperature was 230°C. Fatty acids were identified against the standard solutions of methylated fatty acid esters (Supelco). Analytical data were automatically transformed by the Carlo Erba SP4270 integrator-calculator.

2.4. Fluorescence measurements

Fluorescence measurements were performed according to the method of Schachter et al. [22]. The fluorescent probe 1,6-diphenyl,1,3,5-hexatriene (DPH) was mixed with membranes and incubated at 37°C for 30 min. The measurements were made on the Perkin-Elmer (USA) MPF66 spectrofluorometer. The polarization (P) values were obtained by use the following equation:

$$P = \frac{I_v - I_p(G)}{I_v + I_p(G)}$$

where G represents the instrumental correction factor and I_v and I_p are the intensities of the emitted light oriented, respectively, parallelly and perpendicularly to the plane of polarized excitation light. Fluorescence polarization is inversely related to the rotational rate of the probe which is known to be embedded in the deeper part of the membrane: a decrease in P level, therefore, indicated a greater mobility of DPH and an increase in membrane fluidity.

2.5. EPR measurements

Syncytiotrophoblast membranes were collected, washed three times and then resuspended in buffer A (0.8% NaCl, 0.02% KCl, 0.024% KH_2PO_4 (pH 7.4)). Supernatant after the third wash in buffer A gave no paramagnetic signal. The spin labels used were two stearic acid derivatives (5-nitroxystearate and 16-nitroxystearate) with the paramagnetic (nitroxide) group at C-5 and C-16 of the aliphatic chains, respectively [23,24]. The spin labels were dissolved in absolute ethanol at a final concentration 10 mM and kept at −20°C until the experiment was performed. The spin labels were added to membrane suspensions, vortexed and incubated for 2 h at 37°C. The spin label/phospholipid molar ratio was about 1:180 and the incorporation of spin label was complete (no free spin label signal was detectable). 20 μl of 0.04 M $K_3Fe(CN)_6$

Table 1
Na^+/K^+-ATPase activity of syncytiotrophoblast plasma membranes obtained from term and early placentas

	Term placentas	Early placenta
Na^+/K^+-ATPase	1.35 ± 0.09	1.95 ± 0.05 *
K_m (mM ATP)	1.93 ± 0.35	1.59 ± 0.20
V_{max}	0.87 ± 0.07 *	1.25 ± 0.08

The Na^+/K^+-ATPase activity values were expressed as μmol P_i per mg of membrane proteins per h. * $p < 0.001$.

in buffer A was added, the suspension was vortexed and added to capillary tubes. EPR spectra were recorded at 37°C in a Varian E-4 EPR Spectrometer using a microwave frequency of 9.52 GHz; other parameters were as follows: receiver gain 4×10; modulation amplitude 2G; time constant, 0.5 s; time-course, 4 min. Changes in the mobility of spin labels were reflected as changes in the line widths and separations of peaks in the EPR spectra [25]. In general, the ordering parameter was related to the position and separation (hyperfine splitting) of the resonance lines, while the dynamic properties were reflected by their line width. The order parameter S_n was calculated from the EPR spectrum, according to the formula of Gaffney [26].

2.6. Freeze-fracture studies

Placenta syncytiotrophoblast membranes were fixed in 1% glutaraldehyde for 20 min, cryoprotected in 27% glycerol and, after 15 min, quick-frozen in freon 22 and transferred into liquid nitrogen. The samples were fractured in a Balzers BAF 301 apparatus at −113°C without etching, shadowed with 22 Å of carbon-platinum replica at 45°, followed by a carbon deposition. The replicas were digested in chlorox at 20°C for 50 min, rinsed in double-distilled water and mounted on rhodio-copper grids [27–29]. Observations were made using a Philips CM 10 electron microscope operating at 80 kV. The morphometric measurements and the distribution of IMPs were carried out, by the methods of Roos et al. and Maraldi et al. [30,31], respectively. The distribution factor DF was calculated as the ratio between the variance of number of particles and the mean of number of particles per unit area. In this way, DF gives values of 1 if the particles in the sample are Poisson distributed, < 1 if they are non-random regularly distributed, and > 1 if IMPs are clustered. Membrane fracture faces are described according to a system of nomenclature that refers to exoplasmic (E) and protoplasmic (P) fracture faces [32].

Table 2
Polarization P parameters of the DPH and S_n order parameters of 5- and 16-NS in syncytiotrophoblast plasma membranes obtained from term and early placentas

Parameter	Term placentas	Early placentas	
P	0.260 ± 0.003	0.200 ± 0.002	$p < 0.001$
5-NS	0.635 ± 0.007	0.595 ± 0.009	$p < 0.001$
16-NS	0.498 ± 0.002	0.470 ± 0.005	$p < 0.05$

Table 3
Cholesterol to phospholipid ratio, insaturated to saturated ratio of syncytiotrophoblast plasma membranes obtained from term and early placentas

Parameter	Term placentas	Early placentas	
C/P	0.27 ± 0.001	0.12 ± 0.003	$p < 0.001$
Uns/Sat	0.28 ± 0.006	0.35 ± 0.004	$p < 0.005$

3. Results

As shown in Table 1 the Na^+/K^+-ATPase activity is significantly decreased in the syncytiotrophoblast plasma membrane obtained from term placentas in respect to the early ones (being 1.95 ± 0.05 for early

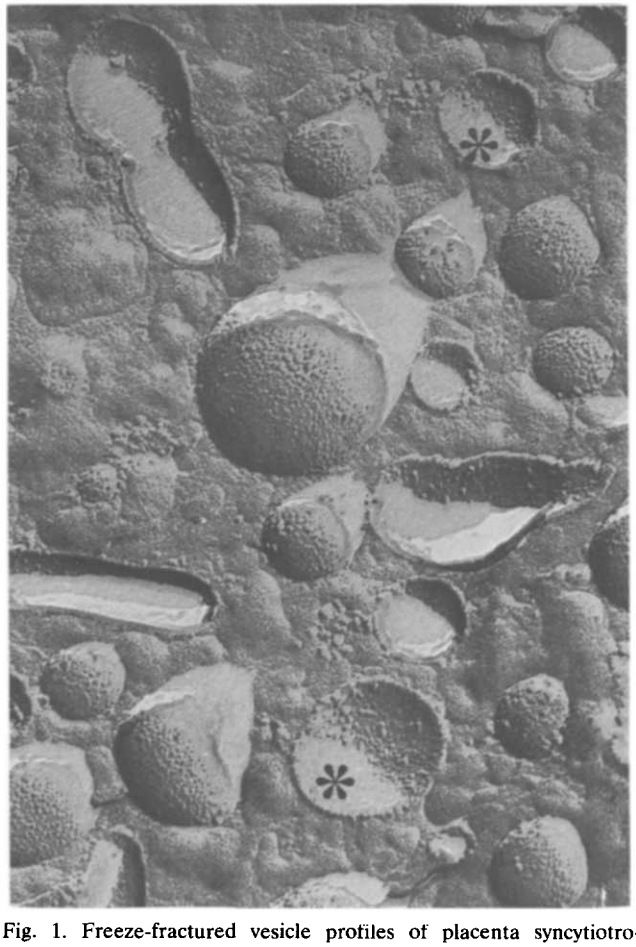

Fig. 1. Freeze-fractured vesicle profiles of placenta syncytiotrophoblast membrane at term of normal pregnancies. IMPs on E face (*) are randomly distributed. (×96000).

Table 4
Number, diameter and distribution factor (±S.D.) of intramembrane particles in a freeze-fracture replica on P and E faces of microvillus plasma membrane obtained from both term (T) and early (E) placentas

	P face			E face		
	Number (N IMP/μm^2)	Diameter (nm)	D.F. V	Number (N IMP/μm^2)	Diameter (nm)	D.F. V
T	1334.66	11.50	2.02	1096.66	12.25	0.45
S.D.	191.33	0.53	0.60	286.67	0.53	0.09
E	1496.19	9.56	11.44	1036.80	9.49 *	5.62 *
S.D.	150.74	0.20	4.96	30.55	0.53	0.56

* $p < 0.05$.

and 1.35 ± 0.09 μmol P_i/mg of membrane protein per h for term placentas, $p < 0.001$).

A marked change in the enzymatic properties of Na^+/K^+-ATPase was found in microvillus plasma membrane from term placentas, with a significant variation of maximum velocity (V_{max}) (Table 1).

As shown in Table 2 the microviscosity, evaluated by the P parameter of DPH and S_n parameters of 5- and 16-NS, is increased in the term compared to the early placenta.

This microviscosity increase is accompained, as shown in Table 3, by an increased cholesterol to phospholipids ratio in term placentas, while there is a decrease in the unsaturated to saturated fatty acid ratio. As follows from morphological studies, the number of intramembrane particles was unchanged both in the P face and in the E face in membranes from both the term and early placentas. A decreased mean diameter in the E face was observed in the early placentas as compared to the term placentas (being 9.49 ± 0.53 for the early placentas and 12.25 ± 0.53 for the term placentas, $p < 0.05$). The distribution factor DF, which indicates the particle aggregation state, increased in the E face in the early placenta as compared to the term one (being 5.62 ± 0.56 for the early placentas and 0.45 ± 0.09 for the term, $p < 0.05$) (Table 4; Figs. 1 and 2).

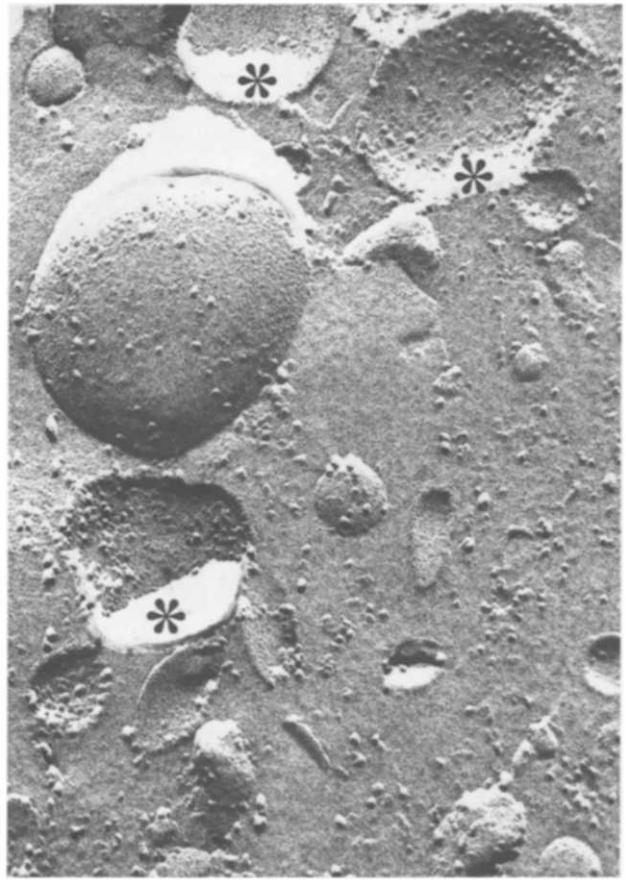

Fig. 2. Freeze-fractured vesicle profiles of placenta syncytiotrophoblast membrane at 10 weeks of gestational age. Note the cluster distribution of IMPs on E face (*). (×96000).

4. Discussion

The placenta is a primary site of nutrient absorption and is essential for the life of the fetus. The uptake of the nutrients needs the syncytiotrophoblast plasma membranes which etablish the contact between mother and fetus. This membrane has been extensively studied in full term placenta [33–35]. However, no extensive studies had been performed on the development of this membrane.

The present study shows that the Na^+/K^+-ATPase activity decreases during pregnancy. This is particularly interesting when it is considered that activities of several enzymes were described as lowered during gestation [36,37].

In the present study the Na^+/K^+-ATPase of microvillus plasma membranes from term placentas showed modifications of V_{max}, suggesting an uncompetitive inhibition of the enzyme. Uncompetitive inhibition affects maximum velocity, being caused by a delay in the splitting of the substrate-enzyme complex or by the presence of enzymatic species which are unable by themselves to combine with substrate [17]. Therefore, the hypothesis of a compositional or conformational perturbation of the Na^+/K^+-ATPase-membrane complex in term placentas is in agreement with the modification of V_{max} observed in this work.

Na^+/K^+-ATPase is an enzyme consisting of subunits which interact during the cycle of the enzyme activity. The lipids of the membrane provide an environment with a fluidity that makes changes possible in the quaternary structure and in the spatial orientation of the subunits during the enzymatic cycle [38–40].

Changes in the interactions between the enzymatic subunits of the Na^+/K^+-ATPase in syncytiotro-

phoblast plasma membranes from term placentas might be expected on the basis of the kinetic study and the freeze-fracture results. In fact, the increased mean diameter of IMPs in the E face of term placentas suggests that polymeric proteins may be associated into larger complexes. The different behaviour of IMPs in the E face of the membrane in term placentas might be explained on the basis of the modified organization of phospholipids in the outer leaflet of the membrane. Moreover, the IMPs of the microvillous plasma membranes from term placentas appear less clustered as compared to those obtained from early ones. The aggregation of IMPs, which represent integral protein constituents [41,42], may derive from different functional conditions [43,44], being also controlled by the spectrin subplasmalemmal network [45]. However, the IMP distribution is greatly influenced by the state of lipid organization of the membrane [16,46,47].

The activity of membrane-bound enzymes is, in most cases, dependent on, or modulated by, the membrane lipid phase [38,39]. In previous studies on syncytiotrophoblast cell culture obtained from term placentas a correlation was found between the changes in the Na^+/K^+-ATPase activity and the modifications in membrane fluidity [46]. The present data on the kinetics of membrane Na^+/K^+-ATPase and membrane fluidity give further, even if indirect, support to this hypothesis.

In conclusion, the present biochemical morphological study shows that a deep modification of the membrane is at the basis of the syncytiotrophoblast plasma membrane development.

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