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Localization of human placental glucose transporter 1 during pregnancy. An immunohistochemical study

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Summary. To elucidate the potential roles of glucose transporter 1 (GLUT1) in human placenta during pregnancy, we examined the localization of GLUT1 in human placenta at various stages by immunohistochemistry with an anti-GLUT1 antibody by use of both light and electron microscopy. Specific staining for GLUT1 was localized on the apical brush border and along the basal plasma membrane of the syncytiotrophoblasts. The staining at the apical side was more intense than that at the basal side during the early stages of gestation. In later gestational stages, however, the staining pattern at the apical side became blurred and the staining intensity at the basal side increased. The cytotrophoblasts, seen embedded in the basal part of the syncytiotrophoblasts, seemed to show immunoreactivity for GLUT1 along the plasma membranes at the light-microscopic level. However, immuno-electron microscopic analysis with either pre- or post-embedding methods revealed that specific staining for GLUT1 was hardly observed on the cytotrophoblasts, but the cytotrophoblasts were often surrounded by immunoreactive processes of syncytiotrophoblasts. The blood capillaries and erythrocytes in the stroma of placental villi were always immunoreactive for GLUT1 throughout pregnancy. These findings suggest that GLUT1 may play a vital role in human pregnancy.

Key words: Glucose transporter 1, Placenta, Immunohistochemistry, Human

Introduction

Glucose is the major energy source for embryogenesis, but the mammalian fetus is unable to produce this essential substrate through glyconeogenesis until the late gestational stage (Battaglia and Meschia, 1978; Jones, 1991). Therefore, glucose transfer from the maternal to the fetal circulation is a crucial feature of mammalian development. The placenta plays a key role in this transfer process, which is primarily a function of facilitated glucose transport down a concentration gradient from maternal to fetoplacental compartments (Hay et al., 1984; Johnson and Smith, 1985).

Recently, there has been a rapid progress in elucidating the molecular basis for facilitated glucose transport mechanisms across cell membranes. At least five distinct facilitated diffusion sugar transporters (GLUTs 1-5, named in order of their cloning) have been characterized to date (Mueckler, 1990; Thorens et al., 1990). GLUT1 was originally purified from erythrocyte plasma membrane and cloned from a cDNA library of a human hepatoma cell line, HepG2, and sequenced (Mueckler et al., 1985).

In human placenta, GLUT1 is highly expressed, as revealed by immunoblotting analysis and immunocytochemistry (Takata et al., 1992). However, little is known about its precise localization in placental tissue during pregnancy. Since this knowledge may clarify the regulatory mechanism governing glucose transfer from maternal to fetoplacental components, we investigated GLUT1 protein expression in human placenta throughout pregnancy by immunocytochemistry at both light and electron microscopic levels using a specific antibody against GLUT1.

Materials and methods

Tissue preparation

Thirty-five fresh human chorionic and placental tissues were collected at various gestational stages in normal pregnancy (early; 5-12 weeks: 20 samples, middle; 14-20 weeks: 8 samples, term; 37-40 weeks: 7 samples). Informed consent was obtained from all patients. The materials were immediately cut into small pieces (about 1 cm^3) and thoroughly washed in saline to remove blood. They were then immersed in a fixative consisting of 4% paraformaldehyde (PF) and 0.5% glutaraldehyde in 0.1M phosphate buffer (PB) (pH 7.4)

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for 3 h at 4 ºC, and then postfixed overnight in 0.1M PB containing 4% PF at 4 ºC. After fixation, the tissue blocks were incubated in 0.1M PB containing 10% melting gelatin for 4 h at 37 ºC and chilled in ice. Then, gelatin-embedded specimens were immersed in 0.1M PB containing 15% sucrose at 4 ºC overnight.

Pre-embedding immunohistochemical DAB staining

The specimens were frozen rapidly with CO_2 gas and sectioned at a thickness of 7-8 µm with a cryostat, mounted on gelatin-coated glass slides, and air-dried. Immunohistochemical staining for GLUT1 was carried out on these cryostat sections; immunohistochemical procedures have been described elsewhere (Yoshimoto et al., 1990). Briefly, the cryostat sections were incubated with rabbit anti-GLUT1 antibody (Transformation Research, Inc., Framingam, MA) at a dilution of 1:500 in 0.1M phosphate-buffered saline containing 0.3% Triton X-100 (PBST) and 2% bovine serum albumin (BSA) overnight at 4 ºC. They were then rinsed in PBST and incubated with biotinylated anti-rabbit immunoglobulin (Vector Lab. Burlingame, USA) at a dilution of 1:500 in 0.1M PBST for 2 h at room temperature. After several rinses in PBST, these sections were incubated with avidin-biotin-peroxidase complex (Vectastain ABC kit; Vector Lab. Burlingame, USA) at a dilution of 1:500 in PBST for 90 min at room temperature. The blue-black color of positive reactions was developed in a solution of 0.025% 3,3'-diaminobenzidine-4HCl (DAB) (Dojinkagaku, Kumamoto, Japan) containing 0.0003% H_2O_2 and 1% nickel ammonium sulfate in 0.05M Tris-HCl buffer (pH 7.6) for 15 min at room temperature. The specificity of the antibody for GLUT1 has been described elsewhere (Birnbaum et al., 1986). For examination under a light microscope, the sections were dehydrated and coverslipped with Entellan (Merck Darmstadt, Frankfurt, Germany).

For examination under an electron microscope, sections were processed further after immunoperoxidase staining as described above. The sections were then postfixed with a solution of 2% OsO_4 in 0.1M PB for 30 min at room temperature (RT), and after washing with H_2O and dehydration through a graded series of ethanol (EtOH) and propylene oxide, they were embedded in epoxy resin. Ultra-thin sections were cut on an ultramicrotome (Reichert-Jung Ultracut E, Austria) and picked up on collodion-coated copper grids (Veco 150, Okenshoji Co., Ltd., Japan). The grids with ultrathin sections were stained with 2% uranyl acetate and 0.1% lead citrate each for 2 min at RT, washed with H_2O and examined in an electron microscope (H-7100 Hitachi Co., Ltd, Tokyo, Japan).

In the control experiments, the anti-GLUT1 antibody pre-absorbed with the GLUT1 peptide at the concentration of 0.2 mg/ml was substituted for the antibody. The GLUT1 peptide was kindly provided by Dr. Y. Oka at the 3rd Department of Internal Medicine, Yamaguchi University, School of Medicine, Japan.

Post-embedding immunogold staining

The specimens (10 weeks of gestation, 3 samples) were sectioned at a thickness of 50 µm with a vibratome. They were washed first for 10 min with PBS and then for 10 min with H_2O. Sections were dehydrated through a graded series of EtOH and a mixture of EtOH and LR Gold Resin (Ted Pella, Inc., CA, USA) according to the following procedure: 1) incubation for 10 min with 50% EtOH at RT; 2) incubation for 15 min with 70% EtOH at -20 ºC; 3) incubation for 15 min with 90% EtOH at -20 ºC; 4) incubation for 1 h with a mixture of 90% EtOH and LR Gold resin at a ratio of 1:1 at -20 ºC; 5) incubation for 3 h with a mixture of 90% EtOH and LR Gold Resin at a ratio of 1:3 at -20 ºC; and 6) incubation overnight with LR Gold Resin at -20 ºC. The sections were incubated for 1 h with LR Gold Resin containing 0.1% Benzil at -20 ºC, then mounted on silicon-coated glass slides, embedded in LR Gold Resin containing 0.1% Benzil and coverslipped on silicon-coated glass slides. The embedded specimens were polymerized for 4 h in an Ultraviolet Cryo Chamber (Pelco®, Ted Pella, Inc., CA, USA) at - 20ºC. The specimens were observed by light microscopy and the placental villi which contained presumed immunopositive cells were dissected out using a razor blade. Ultra-thin sections of the placental villi were cut on an ultramicrotome (Reichert-Jung Ultracut E, Austria), and ultrathin sections were picked up on collodion and carbon-coated nickel grids (Thin Bar grid, Nishin EM Co. Tokyo, Japan).

The nickel grids with ultrathin sections were incubated for 30 min with 3% normal goat serum (Deko, Denmark) dissolved in a reaction buffer containing 0.2% BSA, 0.2% saponin and 0.05% NH_4Cl in 0.1M PBS at RT. The grids were then incubated for 2 h at RT with anti-GLUT1 antibody diluted 1:100 in the same reaction buffer. The sections were washed with 0.1M PBS and incubated for 1.5 h with immuno-gold conjugated goat anti-rabbit IgG (15 nm gold, British BioCell International, UK) diluted 1:40 at RT. Sections were washed with 0.1M PBS and then with H_2O prior to staining with 2% uranyl acetate and 0.1% lead citrate each for 2 min at RT. Sections washed with H_2O were then examined by electron microscopy (H-7100, Hitachi Co., Ltd., Tokyo, Japan).

Results

Light microscopic observations

Specific staining for GLUT1 was observed in three different locations, i.e. trophoblastic layers, blood vessels and erythrocytes in the placental villi, seen as blue-black staining (Fig. 1). In the sections incubated with preabsorbed anti-GLUT1 antibody, no specific staining was observed (data not shown).

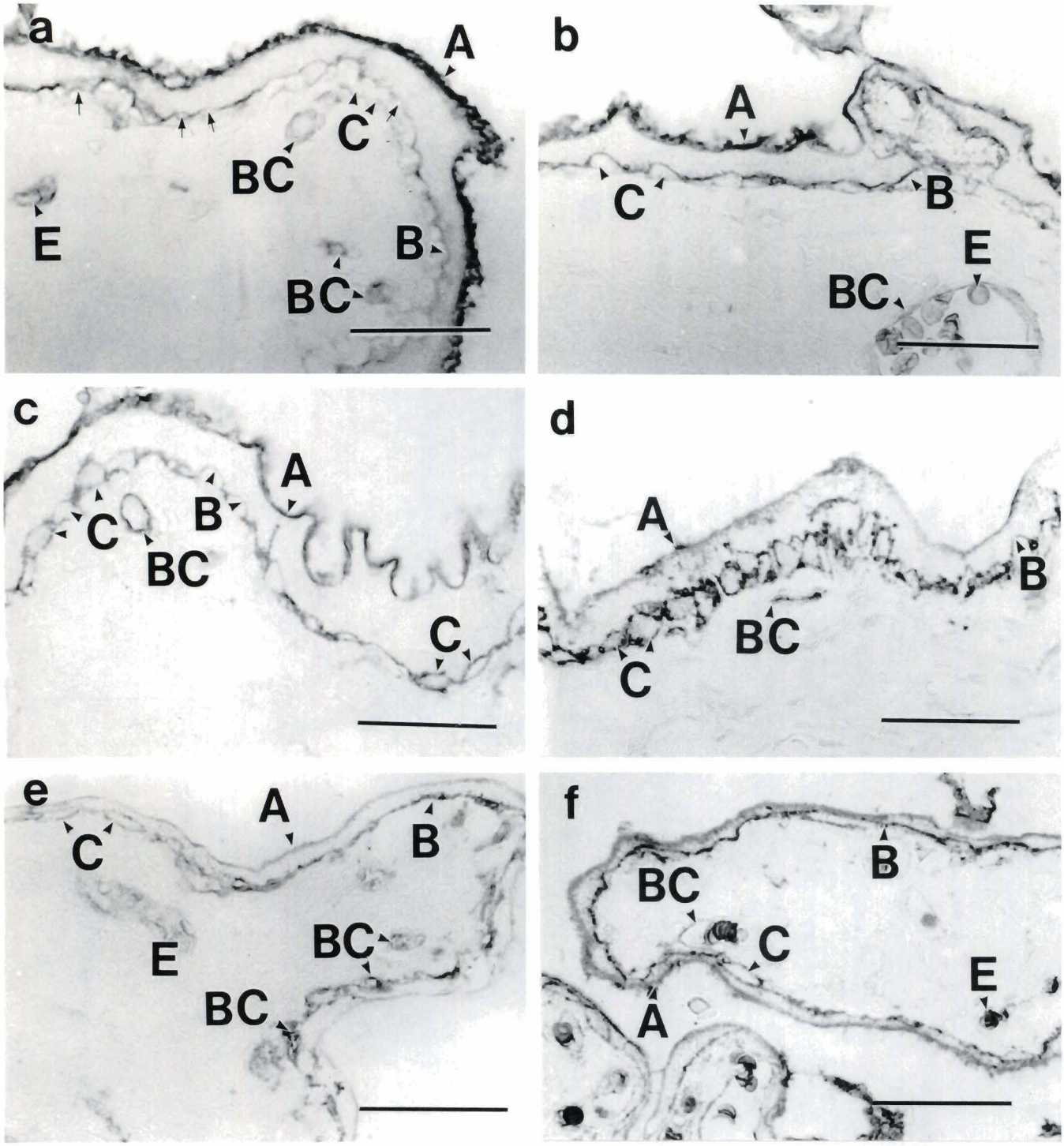

Fig. 1. Immunohistochemical staining for GLUT1 with peroxidase-DAB reaction on cryostat sections (8 µm) of human placental villi. **a.** At 5 weeks of gestation. Note specific staining found on apical (A) and basal (B) membranes of syncytiotrophoblasts. Immunostaining for GLUT1 is also found in blood capillaries (BC) and erythrocytes (E) in stroma. Immunostaining on apical membrane appearing as a continuous line, on irregular basal membrane with many recesses (arrows). Cytotrophoblasts (C) showing negative staining. **b.** At 7 weeks of gestation. Specific staining on apical (A) as well as basal (B) membranes of syncytiotrophoblasts. Also note positive staining in blood capillaries (BC) and erythrocytes (E) in stroma. Specific staining on apical membrane is more intense than on basal membrane. Cytotrophoblasts (C) not completely surrounded by immunostained line. Basal side of cytotrophoblasts is negative for immunostaining. **c.** At 8 weeks of gestation. Specific staining on both apical (A) and basal (B) membranes of syncytitotrophoblasts, and along the surface of cytotrophoblasts (C). Blood capillaries (BC) in stroma also show positive staining. **d.** At 10 weeks of gestation. Intensity of specific staining along the surface of cytotrophoblast (C) appears to be enhanced (compare with Fig. 1c). Both apical (A) and basal (B) membranes of syncytiotrophoblasts, and blood capillaries (BC) show positive staining. Staining on apical membrane (A) of syncytiotrophoblasts becomes blurred. **e.** At mid-gestation (20 weeks of gestation). Specific staining for GLUT1 is seen along both sides of membranes of syncytiotrophoblasts (A: apical membrane; B: basal membrane), and in blood capillaries (BC) and erythrocytes (E) in stroma. Specific staining for GLUT1 is also observed along the surface of cytotrophoblasts (C). No remarkable changes are observed in staining intensity or distribution patterns for GLUT1 (compare with Fig. 1d). **f.** At term (38 weeks of gestation). No remarkable changes are observed in staining intensity or distribution patterns for GLUT1 from mid-gestation through term (compare with Fig. 1e). A: apical membrane of syncytiotrophoblasts; B: basal membrane of syncytiotrophoblasts; C: cytotrophoblast; BC: blood capillaries; E: erythrocyte. Bar: 50 µm. x 440

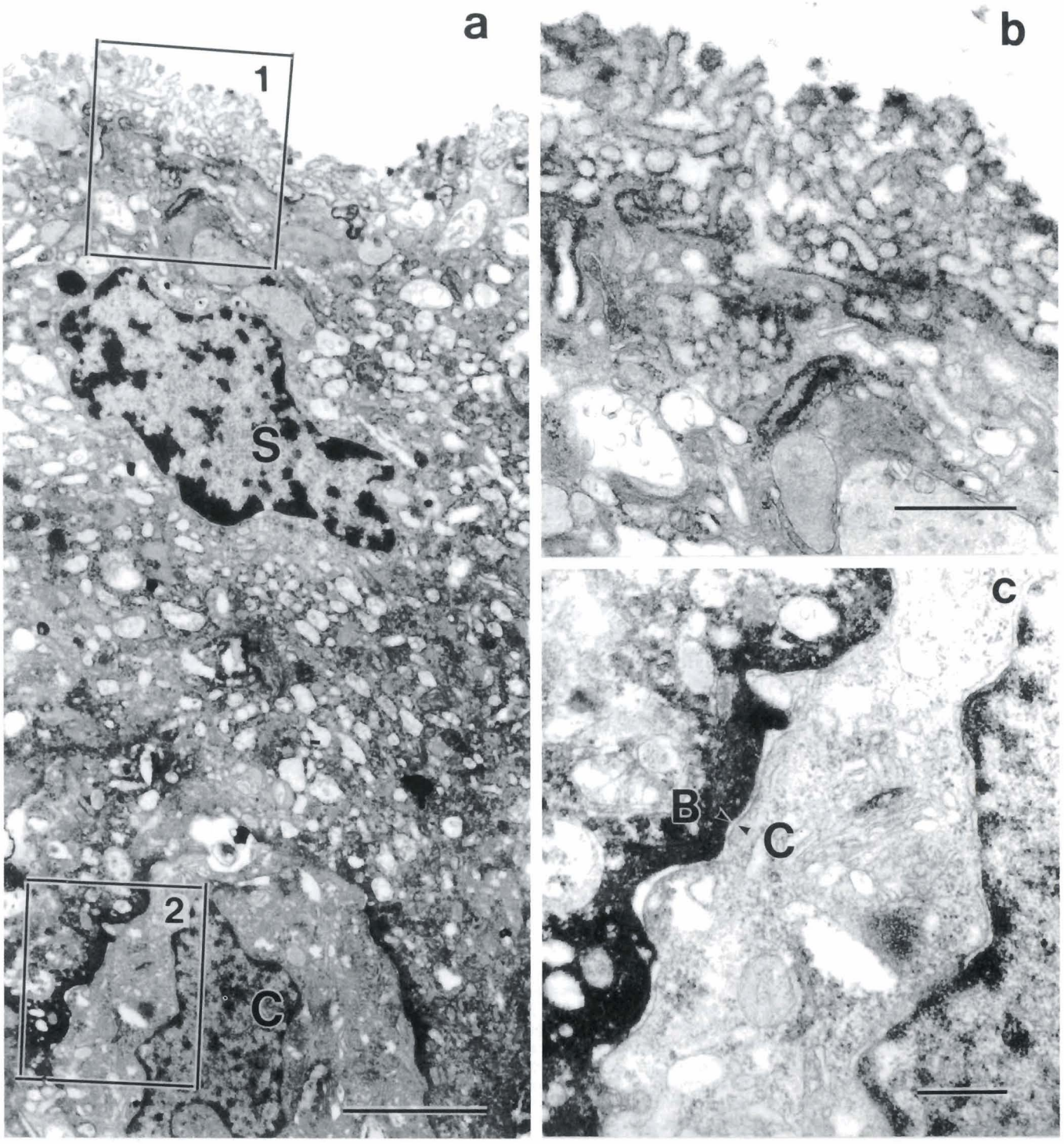

Fig. 2. Immunoelectron microscopic localization of DAB reaction product for GLUT1 in human placental villi at 10 weeks of gestation treated by the pre-embedding method. **a.** Specific deposits are seen on apical microvilli and on the inside of basal membrane of syncytiotrophoblasts, but are hardly seen in cytotrophoblasts. S: syncytiotrophoblast; C: cytotrophoblast. Bar: 2 μm. x 12,600. **b.** Higher magnification of upper framed area in Fig. 2a-1. Specific deposits are seen on apical membrane forming microvilli of syncytiotrophoblasts. x 36,400. Bar: 500 nm. **c.** Higher magnification of lower framed area in Fig. 2a-2. Specific deposits are seen only along basal side of membrane of syncytiotrophoblasts (B), but no notable deposits on the side of cytotrophoblasts (C). x 55,000. Bar: 500 nm.

Trophoblastic layer

Syncytiotrophoblasts

Specific staining was observed at the apical and the basal surface of syncytiotrophoblasts as discrete lines. In chorionic tissue at the early gestational stage, the intensity of specific staining for GLUT1 was prominent on the apical membrane in comparison with that on the basal membrane of the syncytiotrophoblasts (Fig. 1a,b). The staining on the apical membrane appeared to be continuous along the border of the placental villi. However, closer examination revealed that the staining on the basal membrane was irregular and had many recesses or indentations toward the apical membrane. These recesses appeared to be occupied by cytotrophoblasts (Fig. 1a).

After 7 weeks of gestation, the specific staining for GLUT1 at the apical membrane of syncytiotrophoblasts became less intense. The specific staining at the basal membrane tended to have more recesses and showed omega-shaped indentations (Fig. 1b). After 8 weeks of gestation, the GLUT1 staining pattern on the basal membrane of the syncytiotrophoblasts tended to lose the omega-shape and became flat (Fig. 1d-f). No remarkable changes were observed in the staining intensity or distribution patterns for GLUT1 from mid-gestation through term (Fig. 1e,f).

Cytotrophoblasts

Earlier than 7 weeks of gestation, the cytoplasm of cytotrophoblasts was almost completely negative for GLUT1 staining (Fig. 1a,b). At 8-9 weeks of gestation, the cytotrophoblasts were bordered by specific staining for GLUT1 at the basal side of the syncytiotrophoblasts in omega-shaped indentations (Fig. 1c), and staining became more intense from 10 weeks of gestation (Fig. 1d-f).

Blood capillaries and erythrocytes

At 5 weeks of gestation, the blood capillaries were located in the stroma of the villi and predominantly

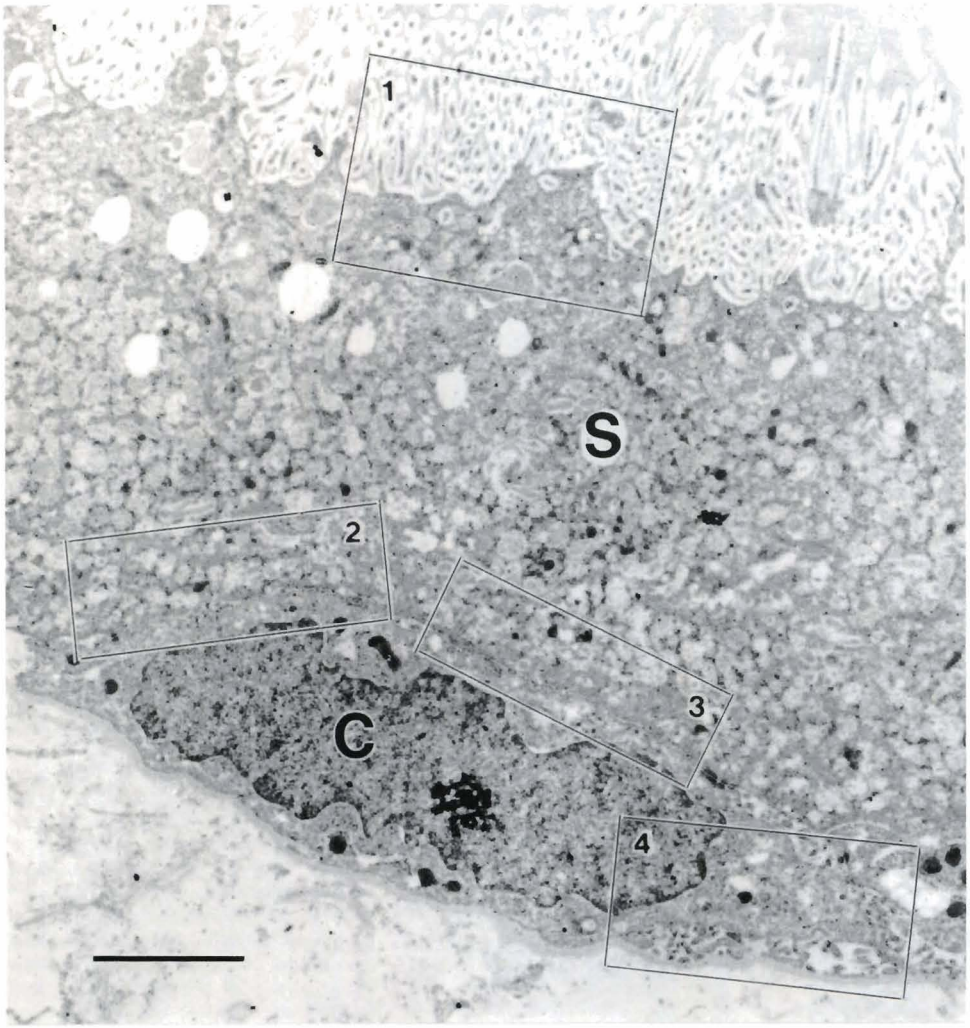

Fig. 3. Immunoelectron microscopic localization of immuno-gold particles staining for GLUT1 in human placental villi at 10 weeks of gestation by post-embedding method. Frames 1-4 indicate the areas shown enlarged in Fig. 4a-d, respectively. S: syncytiotrophoblast; C: cytotrophoblast. x 9,600. Bar: 2 μm.

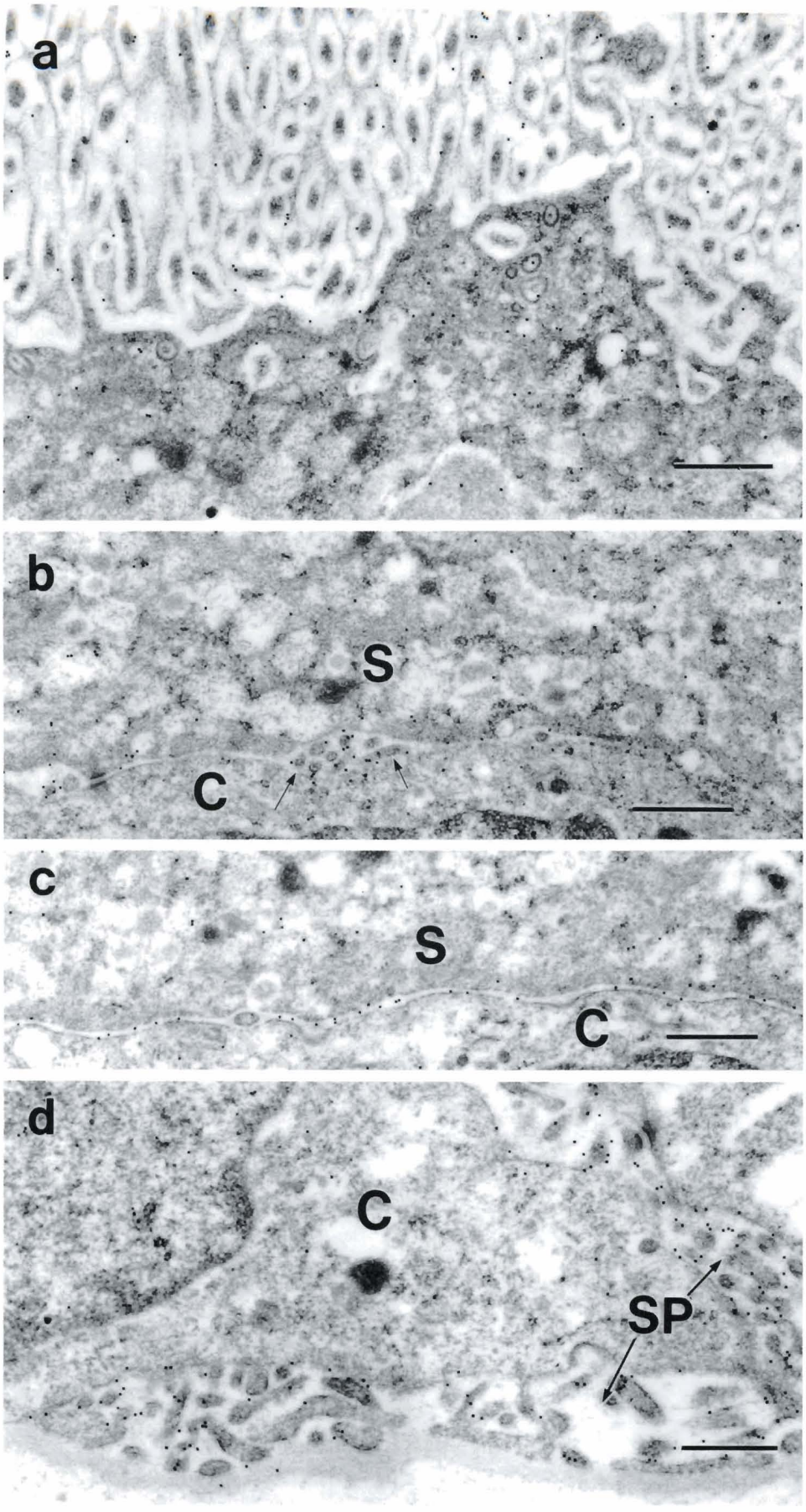

Fig. 4. a. Higher magnification of frame 1 in Fig. 3. Moderate amount of gold particles are situated over microvilli and cytoplasm of apical side of syncytiotrophoblasts. x 30,000. Bar: 500 nm. **b and c.** Higher magnification of frame 2 and 3 in Fig. 3, respectively. Gold particles are most abundant between basal membrane of syncytiotrophoblast (S) and plasma membrane of cytotrophoblast (C) including microvilli of syncytiotrophoblast (arrow). Gold particles are seen in the basal zone of syncytiotrophoblasts (S). Few gold particles are situated over cytotrophoblasts (C). x 30,000. Bar: 500 nm. **d.** Higher magnification of frame 4 in Fig. 3. Processes with many microvilli of syncytiotrophoblast (SP) can be seen to enwrap the basal part of cytotrophoblast (C). Gold particles are abundant over processes of syncytiotrophoblast. Few gold particles are seen over cytotrophoblast. x 30,000. Bar: 500 nm

showed a specific staining for GLUT1 on their basal surface (Fig. 1a). The capillaries appeared to have become dislocated from the center of the stroma to the vicinity of the trophoblasts at the later stages of pregnancy. However, the staining intensity and the pattern of GLUT1 in the blood capillaries showed no distinguishable changes throughout pregnancy (Fig. 1a-f). The erythrocytes in the capillaries were consistently stained with anti-GLUT1 antibody throughout pregnancy (Fig. 1a-f).

Electron microscopic observations

Pre-embedding immunoelectron microscopy with DAB staining for GLUT1

Dense reaction products were only seen in the syncytiotrophoblasts, and specific deposits were seen on the inside of the basal membrane of syncytiotrophoblasts as well as on their apical microvilli. No notable product was seen on the inside of the plasma membrane of cytotrophoblasts (Fig. 2a-c).

Post-embedding immunoelectron microscopy with immuno-gold staining for GLUT1

Gold particles were moderately seen in the syncytiotrophoblasts (Fig. 3). In addition, higher magnification revealed that gold particles were observed particularly over the apical microvilli, and the intercellular space adjacent to cytotrophoblasts (Fig. 4a-d). On the other hand, a small amount of gold particles was observed in the cytotrophoblasts (Fig. 4b-d). The syncytiotrophoblasts extended their GLUT1-immunoreactive processes with a large number of microvilli to enfold the cytotrophoblasts (Fig. 4d).

Discussion

The placenta serves as an organ for exchange of various substances between mother and fetus as the blood-placental barrier. In the human placenta, placental villi are directly bathed in the maternal blood and covered with a single continuous syncytiotrophoblast layer which effectively prevents the mixing of maternal and fetal blood (Dancis and Schneider, 1975; Morriss and Boyd, 1988), and the layer is accompanied by cytotrophoblasts (Benirscke and Kaufmann, 1990). The capillaries are formed in the center of the stroma at the early gestational stages and displaced to the vicinity of the syncytiotrophoblast layer in later stages of pregnancy (Benirschke and Kaufmann, 1990). The syncytiotrophoblast layer serves as a major barrier between maternal and fetal circulation to prevent the entry of undesirable substances and acts as the machinery for the transfer of necessary substances for the fetus and the placenta (Dancis and Schneider, 1975; Morriss and Boyd, 1988). The syncytiotrophoblasts have two opposing domains of their plasma membranes, the apical and basal.

Immunohistochemical studies of GLUT1 in the human placenta have so far been restricted to studies in term placenta, and GLUT1 has been found to be abundant in both syncytiotrophoblasts and cytotrophoblasts (Wheeler and Hinkle, 1985; Takata et al., 1992; Wolf and Desoye, 1993). Our study is the first report of the changes in the localization of GLUT1 from early stages through term of pregnancy. Before 7 weeks of pregnancy, GLUT1 was expressed intensely in the apical side of the syncytiotrophoblast layer. As the gestational weeks progressed, the staining of the apical membrane became wider and less intense. This phenomenon may be the result of elongation of microvilli of the syncytiotrophoblasts (Wislocki and Dempsy, 1955). In contrast, the intensity of GLUT1 staining at the basal side increased with progression of pregnancy. These histochemical findings were roughly in agreement with those of a previous immunoblotting study by Jansson et al. (1993) who reported that the level of GLUT1 expression in the basal membrane increased two-fold in the late second trimester and remained unaltered thereafter to term. However, unlike in their report, a three-fold higher level of GLUT1 in the microvilli than that in the basal membrane at term (Jansson et al., 1993) was not observed in our study.

Our most striking finding was that the cytotrophoblasts were almost negative or only faintly immunoreactive for GLUT1. Light-microscopically, an omega-shaped GLUT1 staining pattern at the basal sides of the syncytiotrophoblast at the early gestational stage might be interpreted as indentations of the cytotrophoblasts toward the apical side of the syncytiotrophoblast layer. This observation was confirmed by our electron microscopic findings in the placenta after 10 weeks of gestation. It is of great interest that both DAB reaction products in the pre-embedding method and gold particles in the post-embedding method were almost always found on the inside of the basal membrane of syncytiotrophoblasts. Although intracellular organellae were not evident in the present study by post-embedding electron immunohistochemical observation, GLUT1 immunoreactivity located inside the basal membrane of the syncytiotrophoblast might be associated with some intracytoplasmic membranous structures. The translocation from cell membrane to GLUT1-loaded intracellular membranes could be responsible for the regulation of glucose transport in the trophoblastic layer, as suggested for the mechanisms of insulin-stimulated glucose transport by GLUT4 in adipocytes. (Gould and Holman, 1993). In fact, gold particles of specific staining for GLUT1 could be observed at the electron microscopic level along the intracellular space. The syncytiotrophoblasts extended processes with intensely-labeled microvilli by gold particles onto the basal faces of cytotrophoblasts and often enwrapped them almost completely. This offers an explanation for the omega-shaped images observed immuno-peroxidase staining. The cytotrophoblasts are enwrapped by the basal

processes of the syncytiotrophoblasts with the progression of gestation (Wislocki and Dempsy, 1955). This may also explain the discrepancy of the result with the previous electron microscopical observation, following the use of the colloidal gold technique, that GLUT1 of the human term placenta is also expressed on the cytotrophoblastic membranes (Takata et al., 1992).

On the other hand, Mouzon et al. (1994) reported that cytotrophoblast cells isolated from human term placenta express GLUT1 at least at the mRNA level. They prepared the purified cytotrophoblast cells according to Kliman et al. who reported that, when placed in culture, the cytotrophoblasts consistently formed aggregates, which subsequently transformed into syncytia within 24-48 h after plating (Kliman et al., 1986). Accordingly, the purified cytotrophoblast cells which they prepared might, in part, already have changed to syncytiotrophoblasts positive for GLUT1. In addition, Douglas and King (1989) pointed out that the isolation procedures and the structural heterogeneity of the placenta itself usually dictated that a mixed tissue population is obtained, which can at least be described as highly enriched for cytotrophoblasts. The «contaminating» cells may include placental macrophages, fibroblasts, endothelial cells and blood elements observed to stain positively for GLUT1 in our study.

The blood glucose level in the fetus is about two-thirds of that in the mother (Morriss and Boyd, 1988; Benirschke and Kaufmann, 1990). However it is almost impossible to accumulate such large amounts of glucose across the trophoblastic layer by a simple diffusion mechanism (Morriss and Boyd, 1988). The trophoblasts may possess a facilitated transport mechanism for glucose following the glucose gradient on the cell membranes (Morriss and Boyd, 1988). GLUT1 is one of the candidate molecules possibly comprising the facilitated transport mechanism, because GLUT1 has been shown to facilitate the transport of glucose down the concentration gradients in both directions across plasma membranes (Farrel et al., 1992). Therefore, it is conceivable that GLUT1 on the apical membrane of the syncytiotrophoblasts facilitates the entry of glucose from maternal blood into their cytoplasm, and GLUT1 associated with the basal plasma membrane may facilitate the glucose transport from the cytoplasm of the syncytiotrophoblasts into the stroma of placental villi.

The existence of abundant GLUT1 on the endothelial and epithelial cells of blood-tissue barriers in various tissues such as blood-brain and blood-ocular barriers has recently been shown by immunoblotting and immunohistochemical techniques (Economides and Nicolaides, 1989; Harik et al., 1990a,b; Takata et al., 1991a,b), and this transporter protein is suggested to play a vital role in the entry of glucose into these firmly guarded tissues. Similar mechanisms serving as blood tissue barriers may also be expected in the placental villi. In this study, we have shown that GLUT1 is abundant in the endothelial cells of the capillaries in the stroma of placental villi. GLUT1, therefore, may facilitate the glucose transfer from the extracellular space to the fetal circulation. On the other hand, the cytotrophoblasts showed almost no immunoreactivity at any stages for GLUT1 in our light and electron microscopic studies. The growth of the placenta, which is most rapid during the first trimester, is driven by multiplication of the cytotrophoblast (Ringler and Strauss, 1990). These observations suggest that GLUT1 in human placenta may be responsible for supplying glucose for fetal growth rather than for placental growth.

Further studies are needed to elucidate the physiological roles and the regulation of GLUT1 expression in human placental tissues.

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