Quantitation and Immunolocalization of Glucose Transporters in the Human Placenta

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SUMMARY

The subcellular distributions of the mammalian passive glucose transporter isoforms GLUT1, GLUT3 and GLUT4,* in the human placenta, were investigated using isoform-specific anti-peptide antibodies. On western blots of both basal and brush-border plasma membranes isolated from the syncytiotrophoblast, antibodies specific for GLUT1 labelled a broad band (apparent Mr 55 000) that co-migrated with the human erythrocyte GLUT1 glucose transporter. In contrast, no labelling was detectable when blots were probed with antibodies specific for the GLUT3 or the GLUT4 isoforms. Densitometric analysis of blots showed that GLUT1 accounts for approximately 90 and 65 per cent of the d-glucose-sensitive cytochalasin B binding sites present in brush-border and basal membranes, respectively. Confocal immunofluorescence microscopy of fixed placental tissue showed that GLUT1 is abundant at both maternal- and fetal-facing surfaces of the syncytiotrophoblast whereas it was undetectable at the fetal capillary endothelium. In parallel experiments, no staining by antibodies against either the GLUT3 or the GLUT4 isoforms was detected in placental tissue. These results indicate that GLUT1 is the major isoform responsible for glucose transfer from mother to fetus. The absence of GLUT4 is consistent with the lack of insulin-sensitive glucose transport across the placenta.

*GLUT1, the erythrocyte/blood-brain barrier glucose transporter; GLUT3, the brain (neuronal) glucose transporter; GLUT4, the muscle/fat glucose transporter.
INTRODUCTION

Both fetus and placenta derive most of their metabolic energy from the oxidation of glucose (Jones, 1991). Numerous studies in the perfused placenta (reviewed in Yudilevich and Wheeler, 1988; Morris and Boyd, 1988) and in plasma membrane vesicles isolated from the human syncytiotrophoblast (Johnson and Smith, 1980, 1985), have shown that the transport of sugars across the placental barrier is mediated by specific carriers localized at both the brush-border (maternal-facing) and the basal (fetal-facing) plasma membranes of the trophoblast. These transporters are present at high densities, show low affinity for glucose, are sodium-independent and can be inhibited by cytochalasin B.

Rapid progress made in recent years has shown that the facilitated-diffusion of sugars across mammalian cell membranes is mediated by a family of homologous polypeptides, the GLUT proteins, which differ in their tissue distributions, hormonal regulation and kinetic properties (Baldwin, 1993). Our group (Barros et al, 1992) and others (Mahler et al, 1992; Shepherd et al, 1992; Takata et al, 1992; Farrell, Young and Pardridge, 1993; Haber et al, 1993; Jansson, Wennergren and Illsley, 1993) have used antibodies in order to identify the GLUT isoforms present in the human placenta. However, the results obtained differ greatly in respect to both the identity and tissue localization of the isoforms detected. For instance, Takata et al (1992) reported similar densities of GLUT1 in both membranes of the trophoblast and in the fetal endothelium. In contrast, we (Barros et al, 1992) and Jansson, Wennergren and Illsley (1993) found that the endothelial density of GLUT1 is negligible when compared with that of the trophoblast. Further confusion arises from the immunocytochemical study by Farrell, Yang and Pardridge (1992) who could not detect GLUT1 in the brush-border membrane but showed similar densities of the transporter in the basal membrane and fetal endothelium. Regardless of the precise localization of GLUT1, it also remains to be demonstrated whether this isoform is indeed the most abundant glucose carrier in the placental barrier.

In the present study, we have performed the first quantitative screening of membranes isolated from the syncytiotrophoblast. The amount of glucose transporters corresponding to GLUT1 was estimated by comparing immunoblots of placental and erythrocyte membranes. This was complemented with GLUT3 and GLUT4 immunoblots and immunostaining of paraformaldehyde-fixed placental sections.

MATERIALS AND METHODS

Preparation and characterization of human placental membrane vesicles
Immediately after delivery, term placentae that were normal in weight and morphology were stored on ice in a polystyrene box for up to 30 min. Syncytiotrophoblast brush-border and basal plasma membranes were then isolated by established techniques (Glazier, Jones and Sibley, 1988; Kelley, Smith and King, 1993). All solutions were supplemented with 0.2 mM phenylmethylsulphonylfluoride (PMSF) to inhibit proteases. When compared with the starting homogenate, the brush-border and basal preparations were, respectively, 17 ± 1.3(20) and 0.76 ± 0.16(6)-fold enriched in the activity of the brush-border marker alkaline phosphatase (Glazier, Jones and Sibley, 1988). The enrichments (fold) of the basal membrane marker [3H]dihydroalprenolol binding (Williams, Jarret and Lefkowitz, 1976) was 1.4 ± 0.16(4) and 22 ± 3(4) for brush-border and basal membranes, respectively. Contamination by erythrocyte membranes in either preparation was <0.5 per cent as determined using the activity of acetylcholinesterase as a red cell marker (Vanderpuye et al, 1988). Membranes
were resuspended in 300 mM mannitol, 5 mM HEPES-Tris, pH 7.4, quickly frozen in liquid nitrogen and stored at −70°C until used. For the normalization of binding and density units, the protein concentration of these membranes was determined by a modification of the method of Lowry et al (1951) using bovine serum albumin as a standard.

**Immunoblots**

Membrane preparations were subjected to sodium dodecyl sulphate 10 per cent polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose filters as described (Davies et al, 1990). After being incubated overnight with the primary antibody, the filters were incubated for 2 h with either a 1:500 dilution of donkey anti-rabbit IgG \(^{125}\)I(F(AB')\(_2\) conjugate (Amersham) or 1:10 000 dilution of donkey anti-rabbit IgG horseradish peroxidase-conjugate (Amersham). The immunoreactive proteins were detected by autoradiography: 6 h to 3 days at −70°C for \(^{125}\)I or 10−120 sec by enhanced chemiluminescence (ECL, Amersham) using Kodak X-Omat AR film and intensifying screens. Western blot densitometry was performed using a standard densitometer or a digital scanner and software developed by Dr S. Bozzo. Both methods yielded similar results.

\[^3\text{H}]\text{cytochalasin B binding}\n
The equilibrium binding of \[^3\text{H}]\text{cytochalasin to placental membranes was assayed by a centrifugation method using either a microcentrifuge or a Beckman airfuge for brush-border and basal membrane vesicles, respectively. Membrane suspensions (0.2−0.5 mg of protein) were incubated with varying concentrations of \[^3\text{H}]\text{cytochalasin B (0.04−1.5 \text{mM}) in the presence of 0.5 M D-sorbitol or D-glucose. The buffer used throughout the assay was 50 mM Tris-HCl pH 7.4 containing 5 mM cytochalasin E to block non-glucose-sensitive cytochalasin B binding sites (Jung and Rampal, 1977). Incubations at 22°C were terminated 30 min later by a 15 min centrifugation at 13 000 g for brush-border membrane vesicles, or at 130 000 g for basal membrane vesicles. Control experiments established that the vesicles were completely sedimented using these conditions. Portions of the supernatant were retained for radioactive determination of the final free \[^3\text{H}]\text{cytochalasin B concentration. The remaining supernatant was removed before assaying the membrane pellet for \[^3\text{H}]\text{activity by liquid scintillation counting. Specific binding is defined as the total binding in the presence of D-sorbitol minus the binding component in the presence of D-glucose. The steady-state D-glucose-sensitive \[^3\text{H}]\text{cytochalasin B binding parameters were determined by fitting the data to a single-site model using the computer program Enzfitter (Elsevier, Biosoft).}\

**Confocal immunocytochemistry**

Human placental cotyledons and umbilical cords obtained shortly after delivery were perfused with 150 mM NaCl, 10 mM NaH\(_2\)PO\(_4\) pH 7.4 [phosphate-buffered saline (PBS)] in order to remove erythrocytes, then with 100 ml 4 per cent paraformaldehyde in PBS. Tissue blocks (5 mm) were left in Zamboni's fixative overnight, washed, and stored at 4°C in 7 per cent sucrose in PBS for up to 4 weeks. Cryostat sections (10 \text{\mu m}) were prepared after embedding the samples in O.C.T. compound (Raymond Lamb, UK) at 40°C. The sections were blocked by incubation for 1 h with 5 per cent swine serum in PBS, followed by overnight incubation with a suitable dilution of primary antibody in PBS. Bound antibody was detected by incubation for 1 h with an 1:80 dilution of swine anti-rabbit IgG fluorescein conjugate.
(Dakopatts) which had been pre-adsorbed with an homogenate of fixed human placental tissue. Finally, sections were washed and mounted in Citifluor anti-fade medium (Citifluor, UK) for examination using a BioRad MRC600 confocal laser scanning microscope.

Materials
Antisera were raised in rabbit against peptides corresponding to the non-conserved C-terminal regions of human GLUT1 (residues 477-492) and GLUT4 (residues 494-509) (Davies et al, 1987, 1990). Antiserum against the C-terminus of human GLUT3 was a gift of Dr G. Gould (Shepherd et al, 1992). Anti-von Willebrand IgG was purchased from Dakopatts, Denmark. Reagent grade chemicals were purchased from Sigma.

Statistical analysis
Data are presented as mean ± s.e. (no. of placentae). The paired Student’s t-test was used to establish significance between groups.

RESULTS

Identification and quantitation of GLUT1 in plasma membranes isolated from the trophoblast
Western blots of both brush-border and basal membranes prepared from human placentae were strongly stained by anti-GLUT1 serum [Figure 1(a)] but not by serum obtained prior to immunization [Figure 1(b)]. The species immunodetected co-migrated with the erythrocyte glucose transporter as a broad band with average M_r of 55 000. The broadness and mobility of this band were similar to those reported previously for the human placental glucose transporter identified by photoaffinity labelling with [3H]cytochalasin B (Johnson and Smith, 1982; Ingermann, Bissonnette and Koch, 1983; Wessling and Pilch, 1984). In some preparations of brush-border membranes, part of the GLUT1 immunoreactivity appeared as a higher M_r species [Figure 1(a)]. This is secondary to the known tendency of this transporter to dimerize and aggregate (Baldwin, Baldwin and Lienhard, 1982).

Densitometric analysis showed that, over the range of GLUT1 protein used, there was a linear relationship between the intensity of the signal on the autoradiogram and the amount of protein present on the blot [e.g. erythrocyte membranes in Figure 1(a)]. Therefore it was possible to compare the relative abundance of the transporter in the two membranes. Such an analysis on four placentae showed that the amount of GLUT1 in the brush-border membranes was slightly but significantly greater than that in the basal membranes [ratio=1.2 ± 0.06(4); P<0.05 in Student’s t-test]. However, this result differed from the findings of cytochalasin B binding experiments, where the total concentration of glucose carriers in the two membranes was found to be essentially identical with steady state binding constants of 0.12 ± 0.02(5) and 0.14 ± 0.02(4) nM for the apparent K_d and B_max values of 90 ± 4(5) and 94 ± 6(4) pmol/mg protein for brush-border and basal membranes, respectively. This result confirms an earlier preliminary investigation (Bustamante, Jarvis and Yudilevich, 1990). In order to investigate this discrepancy further, the absolute concentration of immunologically cross-reactive GLUT1 in the two membranes was determined by comparison of the staining intensity on western blots with that of human erythrocyte membranes of known GLUT1 content, as measured by the concentration of
Figure 1. GLUT1 immunoblot analysis of plasma membranes isolated from human placental trophoblast.
Syncytiotrophoblast plasma membranes isolated from two placentae were electrophoresed on a sodium dodecyl sulphate 10 per cent polyacrylamide gel and blotted onto nitrocellulose. The blot was stained with a 1:1500 dilution of anti-C-terminal GLUT1 serum (a) and pre-immune serum (b). bb, brush-border (50 g of protein); ba, basal (50 g of protein); eryth1 and eryth2, human erythrocyte membranes (2.5 μg of protein and 4.2 μg of protein, respectively). Positions of Mr markers are indicated. GLUT1 densities (arbitrary units) were: bb1, 4018; bb2, 4215; ba1, 3563; ba2, 3687; eryth1, 2618; eryth2, 4188. Results are representative of experiments performed in eight placentae.
Figure 2. Quantitation of placental GLUT1 using erythrocyte GLUT1 as standard. Brush-border membranes from two placentae (○) were immunoblotted alongside increasing amounts of erythrocyte membranes (●). The density of GLUT1 (arbitrary units) is plotted against the total number of glucose transporters as determined by [3H]cytochalasin B binding (see Methods).

[d–glucose-sensitive cytochalasin B binding sites (Jung and Rampal, 1977)]. This type of quantitative experiment is illustrated in Figure 2 for two brush-border preparations. Analysis of membranes prepared from three placentae showed that the concentration of GLUT1 in the brush-border and basal membranes was 82 ± 6(3) pmol/mg and 60 ± 5(3) pmol/mg, respectively. By comparison with the concentration of cytochalasin B binding sites in the two membranes (see above), it can be concluded that GLUT1 accounts for approximately 90 per cent of the glucose transporters present in brush-border membranes and 65 per cent of the glucose transporters present in basal membranes.

Immunoblots of brush-border and basal membranes screened with antibodies specific for GLUT3 (three placentae) and GLUT4 (two placentae) were negative (Figure 3). Brain homogenate and adipocyte membranes were used as positive controls, respectively, and yielded the expected transporter bands (approximately 55 kDa). The <kDa band recognized by the anti GLUT3 antibody in both brain and placental membranes corresponds to actin as shown in western blots by Shepherd et al (1992).

Localization of GLUT1 in the placenta by confocal epifluorescence microscopy

Anti-GLUT1 serum (1:200 dilution) strongly reacted with paraformaldehyde-fixed sections of human placenta [Figure 4(a)]. The signal was found exclusively at both surfaces of the syncytiotrophoblast (bb and ba) and no transporter was detected at the fetal endothelium (fc). An identical pattern of staining was also seen when higher concentrations of serum (up to 1:50 dilution) were used, when antibodies (4 μg/ml) affinity-purified as described by Davies et al (1987) were used, and when the tissue was fixed for only 30 min or in the presence of borohydride (data not shown). Because our results differed from those of Takata et al (1992),
who found abundant GLUT1 in the capillary endothelium of the placenta, we tested the integrity of this structure under our fixation and staining conditions. Figure 4(b) shows that an antibody against von Willebrand factor, an endothelial marker (Bloom, Giddins and Wilks, 1973), strongly labelled the fetal capillary endothelium, demonstrating the preservation in an intact state of this cell layer. Our ability to detect nucleoside transporters immunologically at
Figure 4. Immunolocalization of GLUT1 and von Willebrand factor in a cross-sectioned human chorionic villus. Fixed placental tissue sections (10 μm) were stained with a 1:200 dilution of antiserum against the C-terminus of GLUT1 (a) or with 10 μg/ml of anti-von Willebrand factor IgG (b). Immunofluorescence is shown on the left whereas the corresponding phase contrast image, showing the anatomy of the villi, is shown on the right. bb, brush-border membrane; ba, basal membrane; fc, fetal capillary; m, maternal blood space. Bars=25 μm. These results are representative of experiments performed in three placentae.
the fetal capillary endothelium, and all along the placental vascular tree and umbilical vein in a parallel study (Barros et al, 1995), also indicated that the endothelium was not damaged during fixation. Finally and consistently with the immunoblots, no positive staining was detected in placental sections incubated with either anti-GLUT3 or anti-GLUT4 antibodies (not shown).

DISCUSSION

In the present study, we have investigated the molecular identity and subcellular localization of the placental glucose transporters using antibodies raised against non-conserved sections of three of the known isoforms (GLUT1, GLUT3 and GLUT4). Both immunoblots of isolated plasma membranes and immunocytochemistry of fixed-tissue sections indicate that GLUT1 is a major isoform present in the human placenta. This is consistent with the high abundance of GLUT1 mRNA reported in placental extracts (Bell et al, 1990) and it is also in general agreement with the reports of Takata et al (1992), Farrell, Young and Pardridge (1992), and more recently Jansson, Wennergren and Illesley (1993).

Quantitation of GLUT1 revealed that this isoform accounts for the majority, but not all, of the D-glucose-sensitive cytochalasin B binding sites in the trophoblast plasma membranes, i.e. a minor fraction of the trophoblast glucose transporters corresponds to a different isoform. The abundance of GLUT3 mRNA in the placenta (Bell et al, 1990) would make this isoform a possible candidate to account for the remaining fraction. However, we did not detect GLUT3 in either isolated membranes or fixed tissue, a similar result to that obtained by Jansson, Wennergren and Illesley (1993) and also in agreement with Mahler et al (1992) who investigated placental extracts. In contrast, Shepherd et al (1992) and Haber et al (1993) found GLUT3 in post-nuclear placental membranes, although its abundance was much less than predicted from the high level of GLUT3 mRNA in the tissue (Bell et al, 1990). This discrepancy between GLUT3 mRNA and protein levels might be due to post-translational modification of its C-terminus (e.g. proteolysis) that reduces or prevents its recognition by antibodies. Further work, perhaps with antibodies directed against other domains of GLUT3, is therefore needed to clarify this important point. Recently, Reid et al (1994) reported the presence of GLUT2 and GLUT5 immunoreactivity in human brush-border and trophoblast membranes, respectively. These isoforms, which also transport fructose, might account for a fraction of the non-GLUT1 glucose transporters in the trophoblast.

Our inability to detect GLUT1 in the capillary endothelium indicates that these cells either do not express GLUT1 or, more likely, that the level at which GLUT1 is expressed was below our detection limit. Similar results were reported by Jansson, Wennergren and Illesley (1993). In contrast Takata et al (1992) reported that similar levels of GLUT1 are present in the trophoblast and endothelium. Having discarded non-specific endothelial damage as a possible source for this difference, we suggest that the expression of GLUT1 in the endothelium is much lower than in the neighbouring syncytiotrophoblast. A putative high density of GLUT1 at the capillary would not have an obvious physiological role because the placental endothelium, devoid of tight junctions and occluding zones, does not represent a significant permeability barrier (Challier and Bintein, 1989).

CONCLUSIONS

In conclusion, the quantitative studies described here have shown, for the first time, that GLUT1 is the predominant facilitated glucose transporter expressed in the human placenta,
accounting for 65 and 90 per cent of the glucose carriers present in basal and brush-border surfaces of the syncytiotrophoblast, respectively. The absence of the 'insulin-sensitive' isoform GLUT4 in the placenta is consistent with the apparent insensitivity of placental glucose transfer to insulin (Urbach et al, 1989).

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