External labelling of glycoproteins from first-trimester human placental microvilli

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The brush-border glycoproteins of first-trimester human placentas were investigated by using two external labelling techniques: (1) sequential digestion with neuraminidase and galactose oxidase, followed by reduction with NaB^3H_4 , which ^3H -labels terminal galactose and galactosamine residues; and (2) sequential treatment with periodate and NaB^3H_4 , which ^3H -labels terminal sialic acid residues. The labelling procedures were performed on intact tissue so that the results would more closely approximate the topography of the brush border *in vivo*. The microvilli were isolated, subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, and the [^3H]glycoproteins detected by fluorography. Densitometer scans of the fluorograms of the [^3H]galactoproteins showed that, under reducing conditions, 90% of the protein-associated radioactivity was incorporated into two glycoproteins. The major [^3H]galactoprotein of early placental microvilli had an estimated molecular mass of 92 kDa (desialylated) and migrated as a diffuse band. A minor 180 kDa glycoprotein was less consistently labelled. No change in the apparent molecular mass of either component was detected in the absence of β -mercaptoethanol, suggesting that the 180 kDa component was not a dimer of the 92 kDa glycoprotein. The remaining 10% of the radioactivity was equally distributed among several minor membrane components. Densitometer scans of the fluorograms of the [^3H]sialoglycoproteins showed that, under either reducing or non-reducing conditions, 90% of the ^3H was preferentially incorporated into the 92–110 kDa region of the gel. Although no distinct bands were visible, the higher-molecular-mass region of this area was always most heavily labelled. A minor 180 kDa glycoprotein was also ^3H -labelled. The pattern of brush-border [^3H]glycoproteins from first-trimester placentas differed markedly from that of term placental microvilli and from placental fibroblast plasma membranes that were ^3H -labelled by identical external labelling techniques. These results indicate that: (1) the glycoprotein determinants of brush-border topography change during pregnancy; (2) within the placenta, the major 92 kDa (desialylated) determinant, which has not been previously described, is unique to the trophoblastic cells.

The syncytiotrophoblastic cells of the human placenta are of foetal origin and come in direct contact with maternal tissues. The outer surface of

these cells is completely covered by a complex network of microvilli, termed the brush border. This has the effect of greatly increasing the surface area of the foetal placenta, through which many substances, including oxygen, glucose (Johnson & Smith, 1980) and amino acids (Ruzyski *et al.*, 1978), are exchanged. The microvillar membrane also binds substances in maternal blood, such as hormones (Whitsett & Lessard, 1978; Fant *et al.*, 1979), transferrin (Wada *et al.*, 1979a; Loh *et al.*, 1980) and γ -globulin (Wood *et al.*, 1978). In

Abbreviations used: SDS, sodium dodecyl sulphate; PMSF, phenylmethanesulphonyl fluoride; [^3H]sialoglycoproteins, sialoglycoproteins labelled by sequential treatment with periodate and NaB^3H_4 ; [^3H]galactoproteins, galactoproteins labelled after sequential digestion with neuraminidase and galactose oxidase by NaB^3H_4 reduction.

addition, the specialized syncytiotrophoblastic cell surface is the site of certain enzymic activities (Carlson *et al.*, 1976) and the secretion of several products, including foetal hormones (Dancis *et al.*, 1979). One of the most important and interesting functions of the microvillus membrane appears to be the protection of the foetus against maternal immunological attack (Currie *et al.*, 1968). It is therefore important to characterize the surface molecules of the trophoblastic brush border in order to shed light on the structural determinants that mediate the many functions of this unique interface.

Although the brush border is a constant feature of the syncytiotrophoblastic cells throughout pregnancy, certain structural aspects of this membrane appear to change markedly during gestation. Term and first-trimester human placentas express two different forms of alkaline phosphatase, which are altered with respect to antigenic determinants and other biochemical properties (Fishman *et al.*, 1976). In addition, the brush border of the early placenta is also rich in glycogen and the enzymes that synthesize and degrade this molecule. In contrast, preparations of microvilli from term placentas contain no demonstrable glycogen (Fisher & Laine, 1981, 1984).

In previous studies, microvilli have been isolated from term placentas, and several surface proteins and glycoproteins have been identified by external labelling techniques (Wada *et al.*, 1977, 1979b; Kelley *et al.*, 1979). Because many morphological and biochemical changes occur within the brush border during pregnancy, we initiated these studies to determine if microvilli isolated from first-trimester human placentas have a different pattern of membrane glycoprotein expression from that of the term placenta.

Experimental

Neuraminidase (from *Vibrio cholera*) and Pronase were purchased from Calbiochem-Behring. Galactose oxidase was obtained from Sigma Chemical Co. and Worthington Biochemicals. NaB^3H_4 (100 mCi/mmol) and ^{14}C -labelled bovine serum albumin (10 $\mu\text{Ci}/\text{mg}$) were from New England Nuclear Corp. X-Omat AR X-ray film was a product of Eastman-Kodak Co. Sephadex G-50 and concanavalin A-Sepharose were obtained from Pharmacia. Bio-Gel P-2 was purchased from Bio-Rad Laboratories. Standard glycopeptides were prepared from transferrin (human; Calbiochem) and fetuin (type III; Sigma). M_r standards for SDS/polyacrylamide-gel electrophoresis were purchased from Sigma and were as follows: lysozyme, M_r 14300; β -lactoglobulin, 18400; PMSF-treated trypsinogen, 24000; ovalbumin,

43000; bovine serum albumin, 66000; phosphorylase b, 97400; and β -galactosidase, 116000. β -Galactosidase and β -hexosaminidase were kindly given by Professor Y.-T. Li and Professor S.-C. Li, Tulane University School of Medicine, New Orleans, LA, U.S.A., and Delta Regional Primate Center, Covington, LA, U.S.A.

Two strains of normal fibroblasts were cultured from first-trimester human placentas. The cells were maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) foetal-calf serum, penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$). After trypsin treatment, the fibroblasts were passaged at regular intervals. Only cultures beyond the eighth passage were studied.

First-trimester human placentas were collected as previously described (Fisher & Laine, 1981). Only placentas that were recovered intact were used for labelling. Term placentas were obtained immediately after delivery, dissected free of any decidua and washed extensively in Dulbecco's phosphate-buffered saline to remove remaining blood. For each experiment, several intact first-trimester human placentas, approx. 10 g wet weight, were labelled. A portion of a term placental cotyledon of equivalent weight was excised at random and labelled simultaneously.

The glycoproteins of the brush border were examined by two external labelling techniques. In the first, terminal galactose and galactosamine residues were ^3H -labelled after sequential digestion with neuraminidase and galactose oxidase by reduction with NaB^3H_4 as described by Gahmberg & Hakomori (1973a,b), except that 2 mM-PMSF was added to all the reaction mixtures. Some of the incubation mixtures were not oxidized before reduction. As an additional control, human red-blood-cell ghosts (prepared by the method of Dodge *et al.*, 1963), and human placental fibroblasts were labelled concurrently. After labelling, a membrane and particulate fraction was prepared from the placental fibroblasts by the procedure of Gottlieb *et al.* (1974). The placental microvilli were immediately isolated by cold saline extraction as described by Smith *et al.* (1977).

In the second technique, the terminal sialic acid residues of brush-border glycoproteins were ^3H -labelled by a modification (Wada *et al.*, 1977) of the periodate-oxidation method of Blumenfeld *et al.* (1972), except that the oxidation was allowed to proceed for 20 min rather than for 10 min. Some of the incubation mixtures were not oxidized before reduction. After labelling, the microvilli were isolated as previously described.

In all cases ^3H -labelled microvilli and ^3H -labelled plasma membranes either were immediately solubilized in loading buffer (with or without β -mercaptoethanol, for SDS/polyacrylamide-gel

electrophoresis) or had their labelled carbohydrate moieties isolated (for preliminary structural characterization and enzymic digestion).

Protein was determined by the method of Lowry *et al.* (1951). SDS/polyacrylamide-gel electrophoresis was performed on 7½% (w/v)-polyacrylamide gels (Laemmli, 1970). Some gels were stained for protein with Coomassie Blue (Segrest & Jackson, 1972). Fluorography was performed by the method of Laskey & Bonner (1975). Fluorograms and Coomassie Blue-stained gels were scanned with a soft laser scanning densitometer (Zienh; LKB).

For carbohydrate analysis, microvilli were delipidated (Krusius *et al.*, 1974) by extracting the membrane pellet with 20 vol. of chloroform/methanol (2:1, v/v). The precipitate was then extracted with 20 vol. of chloroform/methanol (1:2, v/v). [³H]Oligosaccharides were prepared by heating (80°C) the delipidated microvilli with 1 M-NaOH and 4 M-NaBH₄ for 24 h. The reaction mixture was neutralized and desalted on a column of Bio-Gel P-2. The resulting oligosaccharides were then re-N-acetylated (Kornfeld *et al.*, 1979). ³H-labelled bi- and tri-antennary standard glycopeptides were prepared as previously described (Fisher & Laine, 1979). Concanavalin A-Sepharose fractionation of standard and placental [³H]oligosaccharides was performed by the method of Krusius (1976). Glycopeptides were prepared by digestion with Pronase. All gel-permeation columns were eluted with 10% (v/v) acetic acid. Enzyme digests were performed by the method of Li & Li (1972). [³H]Sialoglycopeptides were hydrolysed with mild acid as described by Blumenfeld *et al.* (1972).

Results

Labelling of terminal galactose and galactosamine residues

Term-placental cotyledons were labelled five times by sequential treatment with neuraminidase and galactose oxidase, followed by reduction with NaB³H₄. The maximum radiolabel incorporation was 2 × 10⁴ c.p.m./mg of protein. As a result, detection of the electrophoretically separated ³H-galactoproteins by fluorography was not successful.

Intact first-trimester human placentas and red-blood-cell ghosts were labelled after galactose oxidation a total of five times, and human placental fibroblasts were labelled twice. The isolated plasma membranes from all three cell types showed a consistent radiolabel incorporation of 5 × 10⁶ c.p.m./mg of protein. SDS/polyacrylamide-gel electrophoresis of the membrane preparations and detection of the ³H-proteins by

fluorography gave similar results for all labellings (see Figs. 1–3).

The electrophoretic pattern of [³H]galactoproteins from human red blood cells (Fig. 1, lane A) was identical with that previously reported by other investigators (Gahmberg & Hakomori, 1973a; Gahmberg, 1976). Four major proteins were labelled: Band 3, PAS1 (glycophorin), PAS2 and PAS3 (Fairbanks *et al.*, 1971; Mueller & Morrison, 1974). The apparent molecular masses of these proteins, as demonstrated by SDS/polyacrylamide gel electrophoresis, were calculated by Gahmberg (1976) (see Fig. 1 for details) and were used as standards in this experiment. As a control, a portion of the red-blood-cell ghosts was treated with neuraminidase alone, then reduced with NaB³H₄. Fluorograms from SDS/polyacrylamide

Fig. 1. Fluorogram of SDS/7½% (w/v) polyacrylamide gel of ³H-labelled plasma membranes from three cell types. The samples were reduced before electrophoresis. The human erythrocyte glycoproteins (lane A) were used as molecular-mass standards: Band 3, 100 kDa; PAS1, 85 kDa; PAS2, 48 kDa; PAS3, 24 kDa. A portion of the red cells (lane B) was reduced without exposure to galactose oxidase. The first-trimester trophoblastic microvilli (lane C) contained two [³H]galactoproteins. Placental fibroblasts (lane D) did not contain these proteins, but a 220 kDa component (probably fibronectin) was heavily labelled. The arrow indicates the origin.

gels of this preparation (Fig. 1, lane B) showed no non-specific labelling of glycoproteins, but revealed one band that migrated with the front. This non-specific labelling of membrane lipids has been described previously (Gahmberg & Hakomori, 1973a). In addition, [^3H]oligosaccharides were prepared from the delipidated labelled red-blood-cell ghosts and chromatographed on Sephadex G-50 (results not shown). The distribution of radioactivity exactly corresponded to previously published chromatographic profiles (Sephadex G-50) that were generated by g.l.c. of unlabelled red-blood-cell glycopeptides (Jarnefelt *et al.*, 1978). The electrophoretic pattern of placental fibroblast ^3H -labelled plasma membranes (Fig. 1, lane D) was similar to the results of other studies in which labelling of fibroblasts was by the same technique (Gahmberg & Hakomori, 1973b; Gahmberg *et al.*, 1974; Critchley, 1974). This pattern included a 220kDa glycoprotein that was prominently labelled and which is probably fibronectin.

Fig. 2. Fluorogram of first-trimester placental ^3H -labelled microvilli electrophoresed under non-reducing conditions. The labelled brush-border preparation was electrophoresed in the absence (lane B) of β -mercaptoethanol. Lane A contained ^{14}C -labelled bovine serum albumin. The arrow indicates the origin.

The pattern of [^3H]galactoproteins found in microvilli isolated from labelled intact first-trimester human placentas showed three major bands (Fig. 1, lane C). One migrated as a broad band at the front, which is characteristic of lipid. Under reducing conditions the major [^3H]glycoprotein migrated as a diffuse band with an estimated molecular mass (desialylated) of 92kDa. Owing to this particular experimental protocol, the molecular mass of the intact glycoprotein could not be calculated. A minor band of 180kDa was present at various intensities. Fluorograms from gels of unreduced microvilli preparations (Fig. 2) showed the same [^3H]glycoprotein pattern as those from gels of preparations that contained β -mercaptoethanol.

A portion (50 μg) of labelled first-trimester microvillar protein was electrophoretically separated under reducing conditions and the pattern of Coomassie Blue-stained proteins compared with the fluorograms from the same preparation (Fig. 3). The microvilli stained for protein contained approx. 30 major membrane components, but no prominent bands were seen which co-migrated with either the 92kDa or the 180kDa protein.

Labelling of terminal sialic acid residues

First-trimester and term placentas were labelled after mild periodic acid oxidation followed by NaB^3H_4 reduction a total of five times. The isolated microvilli showed a consistent radiolabel incorporation of 5×10^7 c.p.m./mg of protein. The pattern of [^3H]sialoglycoproteins from early placental microvilli was qualitatively similar (Fig. 4, lane B) to that of first-trimester placental microvilli labelled after sequential digestion with neuraminidase and galactose oxidase followed by NaB^3H_4 reduction (Figs. 1–3): one area of the gel was heavily labelled. Fluorograms of electrophoretically separated [^3H]sialoproteins showed preferential incorporation of ^3H into the 92–110kDa molecular-mass region of the gel. However, this incorporation was into a much more diffuse area than was seen in fluorograms of the major [^3H]galactoprotein previously described. Although no distinct bands were visible, the higher-molecular-mass region of this area was always more heavily labelled. This may indicate that the 92kDa [^3H]galactoprotein is heavily sialylated. However, the possibility that additional membrane components of similar molecular masses were labelled could not be eliminated. The minor component was a 180kDa glycoprotein. This pattern was unchanged in the absence of β -mercaptoethanol (results not shown). If the membranes were not oxidized before reduction, only one band, which migrated with the lipid front, was detected (results not shown). Term-placental

Fig. 3. Comparison of Coomassie Blue-stained and [^3H]-galactoproteins from the same preparation of microvilli. Lane B contained 50 μg of ^3H -labelled membranes and was stained for protein. Lane C contained 50 μg (50000 c.p.m.) of ^3H -labelled membranes and was processed for fluorography. Lane A contained the following protein standards: bovine serum albumin, M_r 66000; ovalbumin, 43000; PMSF-treated trypsinogen, 24000; β -lactoglobulin, 18400; lysozyme, 14300. Lane D contained ^{14}C -labelled bovine serum albumin. All samples were reduced before electrophoresis. The arrow indicates the origin.

Fig. 4. Fluorogram of [^3H]microvilli labelled after mild periodic acid oxidation followed by reduction with NaBH_4 . Samples were reduced before electrophoresis. The arrow indicates the origin. Lane A, ^{14}C -labelled bovine serum albumin; B, first-trimester microvilli (50000 c.p.m.); C, term-placental microvilli (50000 c.p.m.).

[^3H]sialoproteins (Fig. 4, lane C) showed a pattern of radiolabel incorporation quite different from that of early-placental microvilli. Many bands were labelled, but there was no preferential incorporation of ^3H into the 92–110 kDa region of the gel as in early-placental microvilli. A 180 kDa glycoprotein was most prominently labelled.

Carbohydrate analysis

The [^3H]galactose-labelled oligosaccharides were prepared from delipidated first-trimester microvilli and chromatographed on Sephadex G-50 (Fig. 5). Peak A had an estimated M_r of 2100. All the radioactivity was released into the low- M_r

region of the column by digestion with β -galactosidase and β -hexosaminidase (Fig. 6). Treatment with either enzyme alone released approximately half of the radioactivity, indicating that desialylation of peak A probably uncovers both galactose and galactosamine termini. The lower- M_r oligosaccharide (peak B) chromatographed as a single peak on Bio-Gel P-2 with an estimated M_r of 540 (Fig. 7). This peak was sensitive to β -hexosaminidase digestion, implying that the terminal sugar is galactosamine. Thus all the label was incorporated into either galactose or galactosamine. Neither oligosaccharide bound to concanavalin A-Sepharose.

[^3H]Sialoglycopeptides were prepared from delipidated term-placental microvilli. A portion

Fig. 5. Sephadex G-50 chromatography of [^3H]oligosaccharides isolated from delipidated microvilli

The column was 30cm in length. Standard triantennary (fetuin) and biantennary (transferrin) glycopeptides were eluted in fractions 43–47 and 49–53 respectively. V_0 is the void volume, V_i the included volume.

Fig. 6. Digestion of Peak A (see Fig. 5) with β -galactosidase and β -hexosaminidase and re-chromatography on Sephadex G-50

All of the radioactivity was shifted to the low M_r region of the column.

(5×10^4 c.p.m.) was chromatographed on Bio-Gel P-2; a 1×10^5 c.p.m. portion was hydrolysed with mild acid before chromatography. After hydrolysis, more than 90% of the radioactivity was shifted to a lower- M_r peak that chromatographed near the inclusion volume of the column. This suggested that the sialic acid residues were specifically ^3H -labelled (results not shown).

Discussion

Previous studies (Wada *et al.*, 1977, 1979b; Kelley *et al.*, 1979) characterized the external

Fig. 7. Chromatography of Peak B (see Fig. 5) on Bio-Gel P-2

The column was calibrated with a standard mixture of glucose oligosaccharides isolated from a partial hydrolysis of starch. The numbers indicate the sugar residues per oligosaccharide (R/O). Fractions (2ml) were collected. All the radioactively labelled material was sensitive to β -hexosaminidase digestion. V_0 is the void volume, V_i the included volume. ●, Intact material; ○, treated with β -hexosaminidase.

proteins (by lactoperoxidase-catalysed iodination) and sialoglycoproteins (by mild periodate oxidation followed by NaB^3H_4 reduction) of human term placental microvilli. The two groups of investigators differed from one another with regard to the pattern of membrane components that were labelled. By using two-dimensional electrophoresis, Wada *et al.* (1977, 1979b) resolved approx. 20 labelled placental proteins, four of which did not share antigenic determinants with membrane components prepared from either human liver or kidney. The identities of three of the labelled proteins, alkaline phosphatase (68kDa), transferrin (80kDa) and the transferrin receptor (188kDa dimer) were established. By contrast, Kelley *et al.* (1979) found that one major component (69kDa) and several minor proteins were labelled in their microvilli preparation.

These previous studies utilized term-placental microvilli that were isolated before labelling. By contrast, all the labelling procedures we performed used intact placental tissue without any prior disruption of the villus surface. Despite the difference in methodologies, our results concerning the labelling (sialic acid) of the term-placental brush border were similar to those of Wada *et al.* (1977, 1979b) in that we found several bands of

various intensities labelled. As a result, it was impossible to establish that the heavily labelled (92kDa, desialylated; 92–110kDa, sialylated) membrane components of the early-placental brush border were not also labelled in term-placental microvilli. However, it was clear that the degree of labelling of these membrane components varied dramatically between early and term placentas. In contrast, we were unable to label similarly the intact-term-placental microvilli after galactose oxidation. The reasons for this remain obscure, although the structural differences between the early- and the term-placental brush borders that are indicated by our study may be implicated.

The results reported here demonstrate that a 92kDa (desialylated) galactoprotein is a prominent component of the external surface of first-trimester human placental microvilli. A minor component with an estimated molecular mass of 180kDa was not as prominently or consistently labelled. In addition, not all placental cells have these glycoproteins as major plasma-membrane components, as evidenced by the electrophoretic pattern of [³H]glycoproteins from placental fibroblasts labelled by the same technique. The pattern of surface-labelled brush-border glycoproteins is not reflected by Coomassie Blue-stained gels of microvilli from the same preparation. This suggests that these glycoproteins are prominently labelled because of their position in the membrane rather than because of their abundance. However, the lack of staining with Coomassie Blue may be due to a high carbohydrate content, as in the case of glycophorin (PAS1) from human red cells. Additional indirect evidence for heavy glycosylation of the major externally labelled galactoprotein of the early placenta was the preferential incorporation of ³H into an extremely diffuse, higher-*M_r* area of the gel after the labelling of the terminal sialic acid residues of brush-border glycoproteins.

The identity of either microvillar membrane component is presently unknown. The possibility that the 92kDa (desialylated) glycoprotein is transferrin has been eliminated on the basis of molecular mass and oligosaccharide structure. The early-placental glycoproteins appear to carry at least two types of sugar chains, a smaller (probably 'O-linked') moiety and a larger (probably 'N-linked') structure, neither of which bind to concanavalin A-Sepharose. By contrast, 95% of the sugar chains of transferrin have the biantennary 'N-linked' structure and interact with concanavalin A (Krusius *et al.*, 1976; Kerckaert *et al.*, 1979; Kerckaert & Bayard, 1982).

Since transferrin was not labelled in this preparation, the presence of the transferrin receptor (188kDa), with which the protein is associated

in preparations of term-placental brush border (Wada *et al.*, 1979a,b; Seligman *et al.*, 1979; Faulk *et al.*, 1978), was equivocal. However, this possibility has not been excluded. Wada *et al.* (1979a) reported that the transferrin-receptor dimer is present in samples that are inadequately reduced and denatured before electrophoresis, a fact which could account for the variable appearance of the minor [³H]glycoprotein of similar molecular mass described in the present study. However, no concomitant change in the electrophoretic migration of the lower-molecular-mass glycoprotein has been observed. In addition, it is clear that the 92kDa component is not the transferrin-receptor monomer, since no shift of this glycoprotein to a higher molecular mass was seen under non-reducing conditions.

The present study demonstrated that a 92kDa glycoprotein (desialylated) is an important topological determinant of the external surface of first-trimester-human-placental microvilli. The identity of this plasma-membrane component is currently unknown. However, the concentration of this glycoprotein at the periphery of the brush border indicates that it is in an optimal position to interact with the maternal tissues with which the placental microvilli are in intimate contact.

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