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Accumulation of malto-oligosaccharides in the syncytiotrophoblastic cells of first-trimester human placentas

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A cell-surface microvillar fraction that was isolated from the syncytiotrophoblastic cells of first-trimester human placentas was found to contain very high concentrations ($890 \pm 32\mu g$ of hexose/mg of protein) of a class of low-molecular-weight oligosaccharides that were comprised entirely of glucose. T.l.c. and gel filtration showed that the saccharides contained from one to six glucose residues. The structures of the most prominent members of the series, a tetra- and a tri-saccharide, were determined. The anomeric configuration of the glucose residues was α, and methylation linkage analysis gave terminal and 4-linked hexose residues. These malto-oligosaccharides contained one reducing terminus per molecule, indicating that they were free and not bound to other structural elements of the cells. Within the placenta they appeared to be concentrated in the first-trimester trophoblastic cells, since crude membrane and particulate fractions isolated from either term trophoblastic cells or cultured placental fibroblasts did not contain detectable amounts of glucose oligomers. This series of oligosaccharides was similar to the products that are formed when glycogen is degraded by α-amylase in liver homogenates and may be indicative of a similar, highly active enzymic reaction closely associated with the brush border of the syncytiotrophoblastic cells of the first-trimester human placenta. Although the role of these oligosaccharides remains obscure they are probably involved in foetal metabolism.

The syncytiotrophoblastic cells of the human placenta completely cover the villi and lie at the maternal/foetal interface. As such, the syncytiotrophoblastic microvillar membrane, which is constantly bathed by maternal blood, is uniquely positioned to perform many vital functions, including the transfer of important nutrients such as glucose (Johnson & Smith, 1980) and amino acids (Ruzycki *et al.*, 1978) and the binding of maternal hormones (Whitsett & Lessard, 1978; Fant *et al.*, 1979), transferrin (Wada *et al.*, 1979; Loh *et al.*, 1980) and γ-globulin (Wood *et al.*, 1978). In addition, the microvillar membrane protects the foetus against maternal immunological recognition (Currie *et al.*, 1968) and is the site of certain enzymic activities (Carlson *et al.*, 1976), as well as the secretion of foetal hormones (Dancis *et al.*, 1979).

The glycogen content of the human placenta varies severalfold during pregnancy, peaking in the first trimester, then steadily decreasing to a relatively low value at birth (Villee, 1953; Capkova & Jirasek, 1968). This decline may be a reflection of other maturational changes in the placenta, such as the content of RNA and the synthesis of some proteins such as human choriogonadotropin, which also peak during the first trimester (Munro, 1980). However, the decrease in placental glycogen is accompanied by a concomitant increase in the ability of the foetal liver to store glycogen (Capkova & Jirasek, 1968) and to synthesize the enzymes necessary for glycogen metabolism (Kornfeld & Brown, 1962). These simultaneous changes may instead indicate that the first-trimester placenta, in addition to its many other functions, also acts as a surrogate liver to the developing embryo (Bernard, 1859). In this light the placental brush border, which transports glucose from the maternal circulation, is the first embryonic site of glycogen synthesis. Accordingly, the syncytiotrophoblastic cells must contain the enzymes for embryonic glycogen metabolism.

In the present study we have found unusually high concentrations of 1,4-α-glucan oligosaccharides associated with cell-surface microvillar preparations isolated from the syncytiotrophoblastic cells of first-trimester human placentas. The most prominent members of this series were tetra- and trisaccharides, whose carbohydrate structure we have

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determined. Although their specific role in glucose metabolism is not clear, the oligosaccharides may be products of α-amylase activity associated with the syncytiotrophoblastic cells and, as such, related to glycogen metabolism in the placenta. Whether the oligosaccharides in this organ are themselves a storage form of glucose is unknown.

Materials and methods

Isolation of cell-surface fraction from syncytiotrophoblastic cells

First-trimester human placentas were obtained after vacuum aspiration and immediately placed in Dulbecco's (1954) phosphate-buffered saline, pH 7.4. During this and all subsequent procedures before the fracturing of the microvilli the tissue was maintained at 10°C. The placentas were washed in several volumes of phosphate-buffered saline and the foetal portion was dissected free of any remaining decidua. The tissue was transferred to fresh phosphate-buffered saline, 4°C, and the microvilli were fractured from the villous surface by the procedure of Smith *et al.* (1977). Before analysis some of the cell-surface pellets were resuspended in 100 μl of 10% acetic acid and all were stored at −70°C. The phosphate-buffered saline supernatant from the membrane isolation was freeze-dried and stored at 4°C.

Isolation of crude membrane and particulate fraction from cultured placental fibroblasts

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% foetal-calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). After trypsinization, the fibroblasts were passaged at regular intervals. Only cultures beyond the eighth passage were studied. A membrane and particulate fraction was isolated from the placental fibroblasts by the procedure of Gottlieb *et al.* (1974).

Isolation of malto-oligosaccharides

The cell-surface fraction was solubilized in 1% Triton X-100 (v/v) as previously described (Harrison *et al.*, 1978). After centrifugation at 100000 ***g*** for 90 min, a portion of the supernatant, which contained the soluble components, was removed for protein (Lowry *et al.*, 1951) and hexose (Dubois *et al.*, 1956) determinations. The remainder was chromatographed on Ultrogel AcA-34, eluted with sodium phosphate buffer (0.1 M, pH 7.5), containing 1% Triton X-100. The resulting fractions were monitored for hexose and for protein by fluorescamine (Samejima *et al.*, 1971). Fractions containing hexose were pooled and the Triton X-100 was removed by stirring with SM-2 Bio-Beads (Holloway, 1973). The molecular weight of the resulting oligosaccharides was estimated by gel-permeation chromatography on a Bio-Gel P-2 (−400 mesh) column (60 cm × 2.5 cm) eluted with 10% acetic acid. A series of malto-oligosaccharides isolated from a partial hydrolysis of starch was used as the molecular-weight standards in this and subsequent experiments.

Alternatively, the isolated cell-surface fractions, which had been frozen in 10% acetic acid were brought to 4°C, transferred to a Dounce homogenizer and any aggregates suspended by 40 strokes of a Teflon-coated pestle. The suspension was stirred in 10% acetic acid for 1 h and the insoluble material removed by centrifugation at 100000 ***g*** for 90 min. The supernatant containing the malto-oligosaccharides was chromatographed directly on Bio-Gel P-2. The acetic acid-insoluble pellet was dissolved in Triton X-100 and chromatographed on Ultrogel AcA-34 as previously described.

T.l.c.

A portion (25 nmol) of acetic acid-solubilized oligosaccharides was repeatedly evaporated with methanol under reduced pressure and spotted on a Whatman silica-gel 'high-performance' plate; 5 nmol each of purified malto-oligosaccharides containing from one to five glucose residues were spotted as standards. The plate was developed twice in acetonitrile/water (4:1, v/v) (Hsieh *et al.*, 1978). Peptides were detected with fluorescamine. The purple sugar-containing spots were detected visually after spraying with orcinol, then H_2SO_4, and heating at 100°C for 5 min (Skipski & Barclay, 1969).

Determination of the sugar composition and structure of oligosaccharides

The sugar composition of each fraction (1 ml) that was included on Bio-Gel P-2 was determined after methanolysis by g.l.c. of the trimethylsilyl methyl glycosides (Sweeley & Walker, 1964; Bhatti *et al.*, 1970) on a Shimadzu-Mini 1 gas chromatograph, equipped with a flame ionization detector and a column (6 ft × 2.6 mm) of 3% OV-101 on 100/120 Supelcoport (Supelco Inc., Belefonte, PA, U.S.A.). Carrier gas was N_2 at 40 ml/min. Peaks were integrated with a Spectra-Physics SP-4100 computing integrator.

To determine whether the oligosaccharides were bound to amino acids or free, the tri- and tetrasaccharides, with the appropriate glucose-containing oligosaccharides as standards, were reduced with 0.25 M-$NaBH_4$ in water overnight. After passing each sample over Bio-Rad AG 50 ion-exchange resin to remove Na^+, borate was removed from the supernatant by repeated evaporation under reduced pressure with methanol. The samples were hydro-

lysed in methanolic 1 M-HCl (100°C, 2h), dried, acetylated with pyridine/acetic anhydride (1:1, v/v; 100°C, for 1h) and the glucose/glucitol ratio was determined by g.l.c. on a column (3ft × 2.6mm) of 3% SP 2340 on 100/120 Supelcoport.

The anomeric configuration of the tri- and tetra-saccharides was determined by the method of Hoffman *et al.* (1972), with malto-oligosaccharides and cellobiose as standards. The percentage survival of each anomer after CrO_3 oxidation was determined by g.l.c. of the alditol acetates on a column (6ft × 2.6mm) of 3% OV-275 on 100/200 Chromosorb W AW.

Linkage positions were determined by methylation analysis (Bjorndal *et al.*, 1967, 1970) and detected as previously described (Jarnefelt *et al.*, 1978; Hsieh *et al.*, 1978; Laine, 1980).

Detergent solubilization of placental fibroblast membranes and phosphate-buffered-saline-soluble syncytiotrophoblastic cell-surface fraction

Fibroblast membranes and the phosphate-buffered-saline supernatants were solubilized in Triton X-100 as previously described. Portions of the solubilized components were removed for protein and hexose determination and the remainder chromatographed on Ultrogel AcA-34.

Results

Determination of the concentration and relative molecular weights of hexose-containing molecules in the Triton X-100-solubilized cell-surface fraction

For each experiment the largely vesicular cell-surface microvillar fractions from several isolations were pooled. The Triton-solubilized microvillar fractions from three separate experiments contained an average of $890 \pm 32\,\mu g$ of hexose/mg of protein (mean ± S.D.). The majority of the proteins chromatographed on Ultrogel AcA-34 in the region of the molecular-weight standards bovine serum albumin and ovalbumin. All of the hexose that could be detected by the phenol/H_2SO_4 method co-chromatographed with a mixture of malto-oligosaccharides, consisting of one to five glucose residues, which eluted near the inclusion volume of the column.

Molecular-weight estimation and sugar composition of oligosaccharides

T.l.c. of the acetic acid-soluble cell-surface fraction showed a series of oligosaccharides, the most prominent members of which co-migrated with maltotriose and maltotetraose. Other oligomers, up to six sugar residues in length, were detectable. None of the orcinol-positive spots contained fluorescamine-reactive material.

Low-molecular-weight oligosaccharides that were isolated from either detergent or acetic acid-solubilized pellets gave identical chromatographic patterns of peaks that were within the inclusion volume on Bio-Gel P-2 (Fig. 1). The total sugar content, as well as the composition of each fraction, was determined by g.l.c. of the trimethylsilyl methyl glycosides. All sugar-containing fractions were comprised solely of glucose and greater than 90% of the hexose co-chromatographed with malto-oligosaccharides containing one to six glucose residues. The most abundant oligosaccharides eluted with maltotetraose and maltotriose, whereas glucose, maltose and the penta- and hexa-saccharides were present in smaller amounts. Chromatographic profiles of either detergent- or acetic acid-solubilized oligosaccharides sometimes included a glucose-containing peak (always less than 10% of the total) in the void volume, which has not been further characterized, but is probably glycogen.

The acetic acid-insoluble cell-surface fraction was solubilized in Triton X-100 (1.26mg of protein, 75 μg of hexose) and chromatographed on Ultrogel AcA-34. No hexose could be detected by the phenol/H_2SO_4 method in the lower-molecular-weight region of the chromatogram.

Determination of the structure of the glucose-containing-tetra- and tri-saccharides

The most abundant members of the series of glucose-containing oligomers, the tetra- and the tri-saccharides, were isolated for structural analysis. The oligosaccharides were purified by chromato-

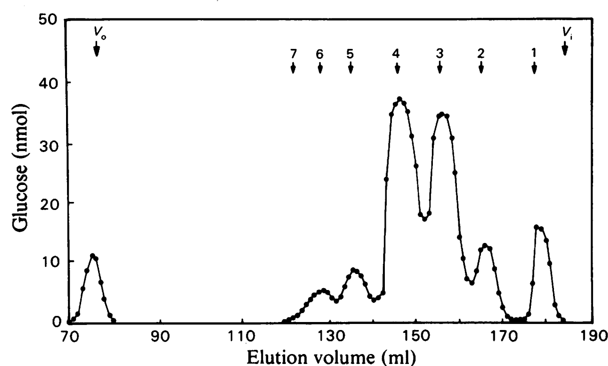

Fig. 1. *Chromatography of acetic acid-solubilized oligosaccharides on Bio-Gel P-2*

The total acetic acid-soluble portion of the cell-surface fraction was chromatographed on Bio-Gel P-2, eluted with 10% acetic acid. Fractions (1 ml) were monitored for sugar composition by g.l.c. The elution volume of standard oligomers is indicated by arrows, with the numbers indicating glucose residues per oligosaccharide. V_0 = void volume; V_i = included volume.

graphy on Bio-Gel P-2 and co-migrated on t.l.c. with the appropriate oligosaccharide standards (data not shown) as single orcinol-positive spots on silica-gel plates developed twice with acetonitrile/water (4:1, v/v).

The anomeric configuration of the oligosaccharides was determined by oxidation with CrO_3, which preferentially degrades the β-anomer (Hoffman *et al.*, 1972). After 1h, only 30% of cellobiose remained, whereas greater than 80% of maltose and maltotriose survived the oxidation. Since nearly 100% of the glucose tetra- and tri-saccharides that were isolated from the syncytiotrophoblastic cells survived the CrO_3 oxidation, they were assigned the α-anomeric configuration.

The tri- and tetra-saccharides were shown to contain one reducing terminus per three and four glucose residues respectively (Fig. 2, Table 1), indicating that the oligosaccharides were free and not attached to amino acids or other structural elements of the syncytiotrophoblastic cells.

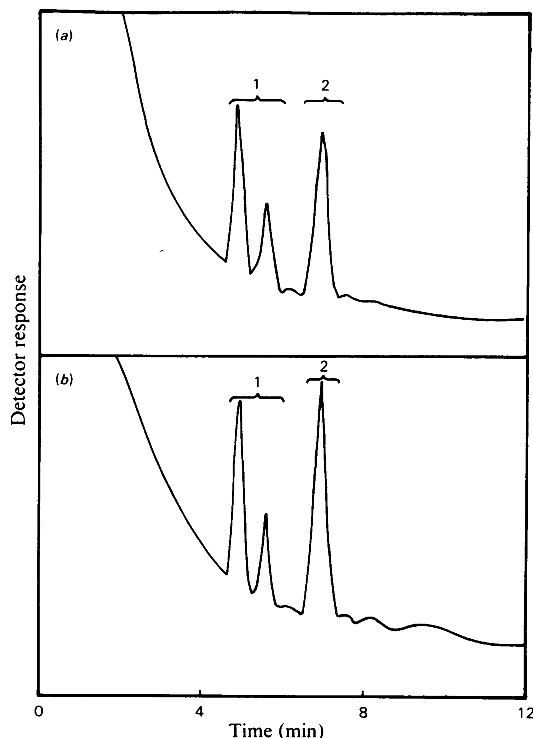

Fig. 2. *Gas-liquid chromatogram of the acetylated hydrolysis products of reduced tetra- (a) and tri-saccharides (b)*
Glucose (1) and glucitol (2) were separated as hexa-acetate derivatives on a 6ft column of SP-2340. The temperature was programmed from 205°C to 250°C at 4°C/min.

Methylation linkage analysis showed that the placental oligosaccharides contained terminal (di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol) and 4-linked (1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitol) hexose residues, which were identified by their retention times as glucose.

Estimation of the hexose content of total placenta

Six placentas were homogenized in phosphate-buffered saline and freeze-dried with the phosphate-buffered-saline supernatant. Protein and hexose were determined as described above.

The total placenta contained 157μg of hexose/mg of protein. The crude membrane and particulate fraction from cultured fibroblasts was solubilized in Triton X-100 and the hexose content was determined to be 78μg/mg of protein. Detergent-solubilized fibroblast preparations, containing a total of 4.5μM-hexose, were chromatographed on Ultrogel AcA-34 and found to contain no low-molecular-weight oligosaccharides. The phosphate-buffered-saline-soluble fraction from the microvilli isolation was treated with Triton X-100 and found to contain 83μg of hexose/mg of protein, none of which was in

Table 1. *$NaBH_4$ reduction of the tetra- and tri-saccharides*
After reduction with $NaBH_4$ the glucose/glucitol ratio was determined for each sample by g.l.c. of the alditol acetates prepared from each sample.

	[Glucose] (nM)	[Glucitol] (nM)	Glucose/ Glucitol
Maltotetraose (from karo)	24.40	8.87	2.75
Maltotriose (from karo)	28.40	13.10	2.16
Placental tetra-saccharide	27.50	9.45	2.90
Placental tri-saccharide	27.70	14.50	1.91

Table 2. *Hexose content of the placenta and some of its tissue compartments*
Results are given as means ± S.E.M. The numbers in parentheses indicate the determinations used for each value.

	Hexose (g/mg of protein)
Whole placenta	157 ± 14 (3)
Supernatant from the cell-surface (microvilli) isolation	83 ± 3 (3)
Syncytiotrophoblastic cell-surface fraction	898 ± 32 (3)
Placental fibroblast membrane and particulate fraction	78 ± 10 (2)

low-molecular-weight oligosaccharides as determined by chromatography on Ultrogel AcA-34.

Discussion

A cell-surface fraction that was isolated from the syncytiotrophoblastic cells of first-trimester human placentas contained nearly equal amounts of hexose and protein. The carbohydrate content of these membranes was several times greater than the values that were reported for a similarly prepared fraction from the syncytiotrophoblastic cells of the human term placenta (Smith & Brush, 1978), which have been found by us and other investigators (Smith *et al.*, 1977) to consist largely of membrane vesicles, with little particulate contamination. However, as previously observed (Smith & Brush, 1978), glucose, which we found to be absent in microvilli prepared from term placentas, was the major saccharide found in preparations of microvilli from first-trimester human placentas.

The elevated carbohydrate content of the cell-surface fraction that was isolated from the first-trimester placentas was due to a class of free malto-oligosaccharides that were comprised of one to six sugar residues, with a tri- and tetra-saccharide as the most prominent members of this series. Although these oligosaccharides fractionated with the microvillar vesicles rather than with the phosphate-buffered-saline supernatant, they were not bound to structural elements of the membrane and were isolated without chemical or enzymic degradation. In addition, the elevated carbohydrate contents were not a characteristic common to all foetal or placental cells. A crude membrane and particulate fraction isolated from cultured fibroblasts that were derived from first-trimester human placentas contained an average of ten times less carbohydrate/mg of protein and the total placenta only double the amount found in the microvillar preparations.

The structure and distribution of the placental oligosaccharides strongly resembled the 1,4-α-glucan series that is formed by homogenized rat liver due to the action of endogenous α-amylase activity (Brosemer & Rutter, 1961). This enzyme rapidly hydrolyses the exterior portions of the glycogen molecule, producing maltotetraose and maltotriose, whereas maltose and the branched species are formed at a slower rate (Olavarria & Torres, 1962). The placental α-amylase, as has been previously shown for the liver enzyme (Brosemer & Rutter, 1961; Mordah *et al.*, 1968), appears to be localized in the microsomal fraction. Presumably the oligosaccharides in the microvillar fraction originate due to the action of the enzyme on glycogen particles that are numerous in the apical portion of the trophoblastic cells of the first-trimester placenta and that, we found, sediment with the microvillar vesicles.

The α-amylase activity of human placental homogenates has been previously described briefly and found to peak during the first trimester (Thakur *et al.*, 1975). These results indicate that the enzyme, its substrate and products are localized within the brush border of the first-trimester human placenta and are absent from fibroblasts comprising the placental stroma. However, the possible occurrence of malto-oligosaccharides in the progenitor cytotrophoblastic cells has not been directly investigated.

The role of the malto-oligosaccharides within the placenta will be clarified by determining whether the placental α-amylase is active in the intact foeto-placental unit or present only in a latent form, as is rat liver α-amylase. In addition, the precise fate of the glucan molecules may be a reflection of the surrogate liver function proposed for the early placenta and therefore may depend on the energy requirements of the placenta and possibly the embryo. The malto-oligosaccharides could thus serve, as has been previously proposed (Mordah *et al.*, 1968), as acceptors for glycogen synthetase, which can use glucose, maltose and maltotriose as acceptors (Salas, 1974; Salas & Larner, 1975). However, the glucan molecules may also act as a source of glucose. Regardless of whether these small glucan molecules are important in meeting the enormous energy demands of the developing embryo, the microvillar membrane fraction of the first-trimester human placenta, which is involved in the transport of maternal glucose and contains enzymes for the synthesis of glycogen, may be an ideal and previously overlooked system in which to study some aspects of the development of glycogen metabolism.

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