Studies of the distribution of glycogen between the inner cell mass and trophoblast cells of mouse embryos*

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Summary. Autoradiographic and histochemical techniques were used to determine the localization of glycogen synthesized during in-vitro culture of preimplantation mouse embryos. During early cleavage embryos accumulated little glycogen and that which was synthesized was spread evenly in the blastomere cytoplasm. However, morula and early blastocyst stages accumulated relatively large amounts of glycogen, especially in the peripheral or trophoblastic cells in comparison to the inner cells or inner-cell-mass cells.

Immunosurgical techniques were used to study the incorporation of radiolabelled glucose into the biochemical pools of inner-cell-mass and trophoblastic cells during culture for 24 h. In general, trophoblastic cells incorporated considerably more isotope than did inner-cell-mass cells, especially into the acid-soluble glycogen fraction. However, inner cell masses isolated on Day 4 of pregnancy incorporated more glucose into acid-soluble glycogen than did inner cells isolated from blastocysts at the end of culture for 24 h in isotope.

Introduction

Synthesis of glycogen by the preimplantation mouse embryo during in-vitro culture has been the subject of considerable study. Morula and blastocyst stages synthesize predominantly acid-soluble glycogen (Brinster, 1969; Pike & Wales, 1982a) whereas early cleavage stages synthesize acid-insoluble glycogen during culture in the presence of glucose (Pike & Wales, 1982a). The utilization of glycogen by embryos is limited under these conditions (Pike & Wales, 1982b) and the accumulation of large quantities of glycogen within the embryos is favoured.

The storage of glycogen within the preimplantation mouse embryo has also been demonstrated histochemically (Thomson & Brinster, 1966) and electron microscopically (Enders, 1971). According to Thomson & Brinster (1966) periodic acid–Schiff (PAS)-positive, diastase-removable material was present in the cleavage-stage embryos up to the morula stage. With the development of the blastocyst this material disappeared from the trophoblastic cells. On the other hand, the electron microscopic study of Enders (1971) has shown an accumulation of glycogen in the trophoblast at the time of implantation in the mouse embryo.

In view of these apparently conflicting results, the cellular localization of glycogen has been studied in the present experiments using autoradiographic, histochemical and immunosurgical techniques.

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Materials and Methods

Embryos were collected from superovulated female mice as described previously (Pike & Wales, 1982a). Modified Krebs–Ringer–bicarbonate medium containing 25 mM-lactate, 0.25 mM-pyruvate, 1 mg bovine serum albumin/ml and antibiotics was used for collection, washing and subsequent culture of embryos (Brinster, 1965). Culture was carried out in droplets under oil as described previously (Pike & Wales, 1982a).

For autoradiographic localization of glycogen the embryos were cultured for 2.5 h in medium containing 0.28 mM-[U- ^{14}C]glucose or [6- ^3H]glucose (sp. act. 1.11 MBq/ μmol for both; Radiochemical Centre, Amersham, U.K.). After culture, the embryos were washed by transfer through 2 changes of medium containing non-radioactive glucose and fixed for 1 h at 4°C in 5% glutaraldehyde in 0.1 M-phosphate buffer (pH 7.3) followed by post-fixing for 1 h at 4°C in 1% OsO_4 in phosphate buffer. Half of the fixed embryos were transferred to a solution of 0.5% amyloglucosidase (EC 3.2.1.3; Grade II, Sigma Chemical Co., St Louis, MO, U.S.A.) in 0.1 M-phosphate buffer (pH 7.3) and incubated at 37°C for 2 h. The other embryos were incubated in phosphate buffer alone. After these treatments the embryos were stained with 1% *p*-phenylenediamine in 70% ethanol for 25 min. They were then pre-embedded in 4% agar for ease of handling (Harris, 1965) and finally embedded in Epon. Sections of 2 μm thickness were dried onto microscope slides and coated with autoradiographic stripping film (Fine Grain Autoradiographic Stripping Plate, A.R.10; Kodak, U.K.). The sections were exposed to the film in the dark for 8 days (^{14}C -labelled) or 6 weeks (^3H -labelled) at -20°C before developing.

The glycogen in blastocysts was also demonstrated histochemically using the PAS technique. Whole blastocysts after culture for 2.5 h in a medium containing 0.28 mM-glucose were placed on microscope slides, fixed and stained as described by Thomson & Brinster (1966). Half of the fixed embryos were treated with 0.5% amyloglucosidase in phosphate buffer at 37°C for 2 h to remove glycogen before staining.

Incorporation of [^{14}C]glucose into inner cell masses and whole blastocysts was studied using the technique of immunosurgery (Solter & Knowles, 1975; Handyside & Barton, 1977). Mouse blastocysts collected 99–100 h after hCG injection were treated for 5–10 min with pronase (110 PUK units; Calbiochem, Los Angeles, CA, U.S.A.) to remove the zona pellucida, then washed through 2 changes of culture medium (2 ml/wash). One third of the washed embryos were transferred into droplets of rabbit anti-mouse serum in basic culture medium (1:5 dilution) and incubated for 30 min at 37°C. After incubation the embryos were washed twice in culture medium to remove excess antiserum and placed in medium containing a 1:10 dilution of guinea-pig serum. When lysis of trophoblast was complete (30–40 min incubation at 37°C) the inner cell masses were recovered, washed twice in basic culture medium and cultured for 24 h in a medium containing 0.28 mM-[^{14}C]glucose (sp. act. 1.11 MBq/ μmol). The inner cell masses were recovered after culture, washed twice in medium containing 0.28 mM non-radioactive glucose and stored at -70°C before fractionation. These samples were designated ICM_1 .

The remaining zona-free blastocysts were cultured for 24 h in radioactive glucose medium similar to that used above. After culture, one half of the embryos were recovered, washed and stored for fractionation. The other blastocysts were subjected to immunosurgery to recover the inner cell masses (ICM_2). To prevent leaching of glucose label from these inner cells during the immunosurgical procedure, the rabbit anti-mouse serum and guinea-pig serum were diluted in radioactive glucose medium identical to that used during culture. The inner cell masses recovered were washed through 2 changes of non-radioactive glucose medium and stored at -70°C before fractionation. The inner cell masses and whole blastocysts obtained from the different treatments were extracted into acid-soluble glycogen, acid-insoluble glycogen, plus non-glycogen acid-soluble and acid-insoluble components using a fractionation procedure previously described (Pike & Wales, 1982a). The non-glycogen acid-insoluble component was further fractionated into RNA, DNA, lipid and protein using a micromodification of the sequential separation procedure of

Shibko, Koivistoinen, Tratnyek, Newhall & Friedman (1967). The radioactivity in each biochemical fraction was assayed using scintillation spectrometry and the label incorporated was determined as pg-atom glucose carbon/inner cell mass or blastocyst.

The cell number in the blastocysts before and after culture and in the inner cell masses isolated from these blastocysts was estimated using the method given by Tarkowski (1966).

Results

Autoradiographic studies

The autoradiographs of embryos labelled with [^{14}C]glucose or [^3H]glucose gave similar results. However, embryos cultured in [^3H]glucose showed more clearly the precise location of labelled glycogen due to the smaller number and spread of developed grains.

At the 2-celled stage a relatively small number of developed grains was found in the autoradiographs (Pl. 1, Figs 1 & 2). When amyloglucosidase-treated and untreated embryos were compared visually, little change in the grain density throughout the blastomeres was observed (Pl. 1, Fig. 2). Therefore, an attempt was made to quantify the grain density in these embryos by counting the grains per unit area in blastomeres of treated and untreated sections. Using this method, a significant effect of enzyme treatment was found ($t_{18} = 3.07$, $P < 0.01$), the grain number/unit area in treated embryos (8.7 ± 0.5) being 30% lower than that in untreated controls (12.9 ± 0.9).

Relatively large accumulations of label were found in the outer cells of morulae and the trophoblastic cells of early blastocysts when compared to the inner cells of these embryos (Pl. 1, Figs 3 & 5). Treatment with amyloglucosidase reduced the density of label in these peripheral cells of morulae and early blastocysts to a level similar to that found in inner cells (Pl. 1, Figs 4 & 6), indicating the removal of glycogen from these cells. In a small number of sections, cells located in association with the inner cell mass, and apparently having no exposure to the exterior, had accumulated considerable quantities of label, thus resembling the outer trophoblast cells (Pl. 2, Fig. 7).

At the 8-celled stage, some embryos exhibited a low and uniform labelling of blastomeres similar to that seen at the 2-celled stage. In others, however, some blastomeres were more heavily labelled (Pl. 2, Fig. 8).

Histochemical studies

Whole blastocysts stained by the PAS technique showed large accumulations of PAS-positive material in the trophoblast cells (Pl. 2, Fig. 9). The embryos that had been treated with amyloglucosidase before staining were devoid of the red stain, indicating that the PAS-positive material was glycogen (Pl. 2, Fig. 10).

Incorporation studies

Direct cell counts on embryos and inner cells indicated that the blastocyst-stage embryos used for the study of isotopic incorporation contained a total of 38.7 ± 1.3 cells (estimated on 6 embryos) of which 17.0 ± 0.6 were found in the inner cell mass (estimated on 6 ICMs). After culture for 24 h *in vitro*, expanded blastocysts and their inner cell masses consisted of 74.0 ± 10.0 (6 embryos) and 24.2 ± 2.9 (6 ICMs) cells respectively.

The data obtained for the incorporation of [^{14}C]glucose into various biochemical fractions of inner cell masses and whole blastocysts following culture in 0.28 mM -[^{14}C]glucose are given in Table 1. The inner cell masses incorporated a relatively small amount of label (7.98 pg-atoms/ICM/24 h) compared to that of whole blastocysts (271.5 pg-atoms/blastocyst/24 h). A major

proportion of the label incorporated into blastocysts was found in the acid-soluble glycogen fraction. When the label accumulated in inner cell masses cultured for 24 h was compared with that of cell masses isolated from cultured blastocysts, the former tissues incorporated slightly more label into all the fractions. However, the difference in the label accumulated into the acid-soluble glycogen between ICM₁ and ICM₂ did not reach the level of statistical significance due to considerable variation between replicates.

Table 1. Incorporation of glucose carbon into various biochemical fractions of mouse inner cell masses and intact blastocysts during culture in a medium containing 0.28 mM-[U-¹⁴C]glucose, 25 mM-lactate and 0.25 mM-pyruvate

Tissue	No. of ICMs or blastocysts/replicate	Glucose carbon (pg atoms/ICM or blastocyst/24 h) incorporated into:						
		Acid-soluble fractions		Acid-insoluble fractions				
		Glycogen	Non-glycogen	Glycogen	RNA	DNA	Lipids	Protein
ICM ₁ *	30	4.4 ± 2.1	3.4 ± 0.4	0.7 ± 0.2	0.5 ± 0.2	0.4 ± 0.1	0.8 ± 0.1	1.2 ± 0.2
ICM ₂ †	22	0.8 ± 0.1	1.2 ± 0.1	0.5 ± 0.0	0.5 ± 0.1	0.4 ± 0.0	0.5 ± 0.2	0.9 ± 0.1
Blastocyst	42	201.3 ± 18.2	20.4 ± 0.8	2.9 ± 0.7	15.1 ± 3.8	5.4 ± 0.2	8.0 ± 0.2	9.6 ± 0.2

* Inner cell masses isolated before culture.

† Inner cell masses isolated from cultured blastocysts.

Values are means ± s.e.m. for 3 replicates.

To compare the metabolic activity of the trophoblast with the inner cell mass, the glucose label incorporated per cell by each cell type was calculated. For this calculation, the data obtained for the incorporation of [¹⁴C]glucose into the whole blastocysts during culture for 24 h and into inner cells isolated from these blastocysts (ICM₂) were used. The glucose label incorporated into the trophoblast was estimated from the difference between the data for the whole blastocysts and that for ICM₂. Using the estimates of cell numbers in inner cell masses and blastocysts the incorporation of label into all the biochemical fractions/cell of each embryonic tissue was calculated. The estimates obtained (Table 2) indicate that the incorporation of glucose label into all the fractions, but in particular into the acid-soluble glycogen fraction, is considerably higher for trophoblastic cells than for inner cells.

PLATE 1

Fig. 1. Autoradiograph of 2-celled mouse embryos labelled with [³H]glucose. The small amount of label is distributed equally throughout the cytoplasm of the blastomeres. × 375.

Fig. 2. Autoradiograph of 2-celled mouse embryos labelled with [³H]glucose followed by treatment with amyloglucosidase to remove glycogen. Little change in the grain density is noticed when compared with Fig. 1. × 375.

Fig. 3. Autoradiograph of mouse morulae and early blastocysts labelled with [³H]glucose. The large accumulations of label in the outer cells or morulae and trophoblast cells of blastocysts are clearly demonstrated. × 250.

Fig. 4. Autoradiograph of embryos at the same stages as used in Fig. 3 but after removal of glycogen with amyloglucosidase. Note the reduction in grain density of peripheral cells to levels similar to those of inner cells. × 200.

Fig. 5. Autoradiograph of mouse morulae and early blastocysts labelled with [¹⁴C]glucose. As in Fig. 3 accumulation of label predominates in the outer cells of morulae and trophoblast cells of early blastocysts. × 200.

Fig. 6. Autoradiograph of embryos at the same stages as used in Fig. 5 but after removal of glycogen with amyloglucosidase. Observations are similar to those in Fig. 4. × 200.

PLATE I

(Facing p. 536)

Fig. 7. Autoradiograph of early mouse blastocysts labelled with [^{14}C]glucose. Inside cells accumulating large amounts of label can be seen in association with the inner cell mass. Two such cells are indicated by the arrows. $\times 250$.

Fig. 8. Autoradiograph of 8-celled mouse embryos labelled with [^{14}C]glucose. In 2 of the embryos, one blastomere is more heavily labelled than the rest. The remaining 3 embryos exhibit uniform low labelling of the blastomeres sectioned. $\times 160$.

Fig. 9. Whole mount of a blastocyst stained by the PAS method. Large accumulations of PAS-positive material are seen in the trophoblast cells of the embryo. $\times 350$.

Fig. 10. Whole mount of a mouse blastocyst stained by the PAS method after treatment with amyloglucosidase which has removed the PAS-positive material from the trophoblast cells (compare with Fig. 9). $\times 350$.

Table 2. Incorporation of glucose carbon into various biochemical fractions of inner cell mass and trophoblastic cells of mouse blastocysts

Component	Glucose carbon (pg atoms/cell/24 h) incorporated into:					
	Acid-soluble fraction		Acid-insoluble fraction			
	Glycogen	Non-glycogen	Glycogen	RNA	DNA	Lipids Protein
Inner cell mass	0.03	0.05	0.02	0.02	0.02	0.02 0.04
Trophoblast*	4.20	0.39	0.05	0.29	0.10	0.15 0.17

* Estimated by difference between incorporation into whole blastocysts and that into ICM₂.

Discussion

The small amounts of label detected by autoradiography in 2-celled embryos in comparison to that at later stages agrees with the results of incorporation studies during preimplantation development of mouse embryos (Brinster, 1969; Pike & Wales, 1982a). The uniform labelling at the two-celled stage suggests that the small amount of acid-insoluble glycogen synthesized at this time (Pike & Wales, 1982a), and which may act as a core or nucleus for the subsequent synthesis of acid-soluble glycogen, is not concentrated in any particular organelle but is distributed throughout the cytoplasm of the blastomeres.

From the autoradiographic, histochemical and biochemical findings at the morula and blastocyst stages it is clear that during differentiation the outer cells of the morula and subsequently the trophoblastic cells of the blastocyst acquire the ability to synthesize large amounts of glycogen. The present observations therefore support the electron microscopic data of Enders (1971) rather than the histochemical data presented by Thomson & Brinster (1966) and show that glycogen is stored in the trophoblast, rather than in the inner cell mass. At the 8-celled stage, differences between blastomeres in some embryos can be found, suggesting that, at about this time, this difference in metabolism is being initiated.

In the autoradiographic studies, it was found that embryos retained some label after treatment with amyloglucosidase. Glucose was incorporated into macromolecules such as nucleic acids, lipids and proteins in both the inner cell mass and trophoblast (Table 2) and this incorporation no doubt accounts for the residual label observed in the embryos after removal of glycogen.

Comparison of the incorporation of label into inner cells isolated before or after incubation for 24 h in [U-¹⁴C]glucose showed that the accumulation of glucose label, especially into acid-soluble glycogen, was greater into inner cells isolated before culture than into those obtained from cultured blastocysts. There was considerable variation between replicates in the amount of acid-soluble glycogen accumulated in inner cells isolated early. Thus it would seem that the inner cell masses isolated before culture contain a variable number of cells that have the biochemical property of accumulating large amounts of acid-soluble glycogen. The present autoradiographic studies (see Pl. 2, Fig. 7) support this suggestion.

The findings of Handyside (1978) and Spindle (1978) indicate that there is complete separation of the cells into inner cell mass and peripheral trophoblast by the expanded blastocyst stage. In the present experiments, therefore, the incorporation into inner cell masses recovered at the end of culture, rather than before exposure to isotope, should more closely reflect the metabolic activity of this cell type. Estimation of the rate of incorporation of glucose into biochemical pools shows that the trophoblastic cells of the late preimplantation embryo are more metabolically active than the cells that form the embryo proper.

In the present experiment the cell number of embryos approximately doubled during the 24-h incubation period, indicating continued viability and division of the cells of the embryos during culture. The ratio of inside/outside cells decreased from 0.78 to 0.49 over this period of

development. Similar reduction in the inside/outside cell ratio of the embryo between early and expanded blastocyst stages has also been observed by Handyside (1978). Considering the high rate of cell division of inside cells in comparison to the outside cells (Barlow, Owen & Graham, 1972) the decrease in the ratio of inside/outside cells adds weight to the suggestion by Handyside (1978) that some outward movement of inside cells occurs during the expansion of the blastocyst. The results presented here indicate that the inside cells that undergo this movement may be cells already biochemically programmed as trophoblastic cells.

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