Increase in the number of glucose carriers in chick fibroblasts during embryo development

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Cytochalasin B (CB) has been used as a tool to ascertain whether the increase in the rate of 3-*O*-methylglucose (3-O-MeG) uptake between the 8th and the 16th day of development in chick embryo fibroblasts could be attributed to an increase in the number of hexose transport carriers. There was a 2–3-fold difference in glucose-specific CB binding between the 8- and the 16-day cells, a difference which is comparable to the previously reported differences in rates of 3-O-MeG uptake. We therefore suggest that glucose-specific CB binding represents binding to the 3-O-MeG carrier and that the increase in the rate of 3-O-MeG uptake from the 8th and the 16th day of development is probably due to an increase in the number of hexose carriers.

Methylglucose (3-O-) uptake *Embryo development* *Cytochalasin B* *Hexose uptake (Chick fibroblast)* *Glucose transport carrier*

1. INTRODUCTION

Various observations now available indicate that a highly active glucose transport system is common to a number of cell types [1–3].

Modifications in the rate of glucose uptake have been reported to accompany cell transformation [1–4] and cell differentiation [5–6]. Using 3-*O*-methylglucose (3-O-MeG), a non-phosphorylatable glucose analog, we previously showed that in chick fibroblasts the rate of hexose uptake increased during embryo development [7]. Kinetic evidence indicates that the apparent V_{max} for hexose uptake is also enhanced. This would reflect an increase in either the number or the mobility of hexose carrier [4,8].

The hexose carriers had to be specifically labelled in order to verify whether their number was increased in older chick embryo fibroblasts. We used the fungal metabolite cytochalasin B (CB), as a molecular marker for the glucose transport protein since it has been shown to inhibit the transmembrane movement of sugars in a variety of cell types [9–12]. In the present report we have investigated the binding of the radiolabelled CB to fibroblasts from chick embryos at various stages of development, the effects of 3-O-MeG on CB binding and the effects of CB binding on 3-O-MeG uptake.

2. MATERIALS AND METHODS

2.1. *Cells*

The fibroblasts were obtained from 8- and 16-days chick embryos (Société Jourdain, France) by the method of Rein and Rubin [13], modified as previously described [14].

2.2. *Cell culture*

Primary monolayer cultures were grown in 16 mm diameter wells in 0.5 ml of Eagle's minimum medium supplemented with 1% glutamine, 1% antibiotics (penicillin, streptomycin) and 10% foetal calf serum. The initial seeding concentration was 10^6 cells/ml (0.5×10^6 cells/well). Cultures were grown in humidified air containing 5% CO_2 at 37°C. A sample of cells was counted in a haemocytometer. 95% of cells were viable as determined by trypan blue exclusion test, and by the

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determination of total lactate dehydrogenase levels in the cells according to the method of Wroblewski and Ladue [15]. Each measurement refers only to viable cells and represents the mean of six samples. The experiments were done on subconfluent cultures (1×10^6 cells/well). Subconfluency in fibroblasts from 8- and 16-day embryos occurs after 48 and 96 h, respectively.

2.3. *Chemicals*

[^{3}H]Cytochalasin B (^{3}H-CB) (specific activity, 11 Ci/mmol) and [3-*O*-^{3}H]methylglucose (specific activity, 6.5–7 Ci/mmol) were obtained from the Radiochemical Centre Amersham (U.K.). Unlabelled CB was obtained from Sigma Chemicals. It was dissolved in 100% ethanol and stored at −20°C.

2.4. *Measurement of 3-O-MeG uptake in the presence of CB*

3-O-MeG uptake was measured in the presence of CB at concentrations ranging from 25 to 5000 nM. These experiments were done as previously reported [7] except that the phosphate-buffered saline (PBS, pH 7.4) contained 0.5% ethanol.

2.5. *^{3}H-CB binding studies*

Subconfluent cultures (10^6 cells/well) were washed three times in PBS containing 0.5% ethanol. The monolayer was overlayed with 0.5 ml of a solution of ^{3}H-CB in PBS (pH 7.4) containing 0.5% ethanol. Incubation time ranged from 30 s to 15 min at room temperature and ^{3}H-CB concentrations ranged from 5 to 2000 nM. The CB binding was studied in the absence and in the presence of a saturating concentration (150 mM) of 3-O-MeG and the latter curve was subtracted from the former to determine the specific binding of CB. After incubation, the monolayer was washed three times with ice-cold PBS (washing took less than 15 s and it caused no significant loss of binding as determined by the counting of the radioactivity in the washing supernatant) and dissolved in 0.5 ml of 0.1 M NaOH before being added to 3 ml scintillation fluid (A.C.S., Amersham). The radioactivity of each sample was counted in a liquid-scintillation spectrometer (Intertechnique SL 30).

3. RESULTS

3.1. *Binding of labelled CB to the fibroblasts*

Direct binding of labelled 3-O-MeG being impossible since flux and efflux processes of 3-O-MeG uptake have been demonstrated to be very fast [7], CB was used as an alternative tool; it has been shown in other cell types [8–12] to bind to glucose carriers in the same way as 3-O-MeG. ^{3}H-CB binding as a function of incubation time is shown in fig.1; the CB concentration was 100 nM. The fibroblasts rapidly bound ^{3}H-CB and at 22°C a plateau was reached within about 10 min. At this stage, 16-day cells had bound twice as much ^{3}H-CB as the 8-day cells. ^{3}H-CB binding was also studied as a function of CB concentration. The data are based on equilibrium binding (15 min) and, within the range of concentrations tested, 16-day fibroblasts proved to bind twice as much ^{3}H-CB as the 8-day fibroblasts. Each experimental point is the average of triplicate determinations. Scatchard analysis [16] of the data gave a concave hyperbolic curve the asymptotic phase of which revealed the presence of unsaturable CB binding sites even at very high concentration of CB. The method of Munck [17] allowed to resolve such Scatchard plots into linear components and led to determine the number of specific CB binding sites (fig.2). Figure 2 indicated that there was only one

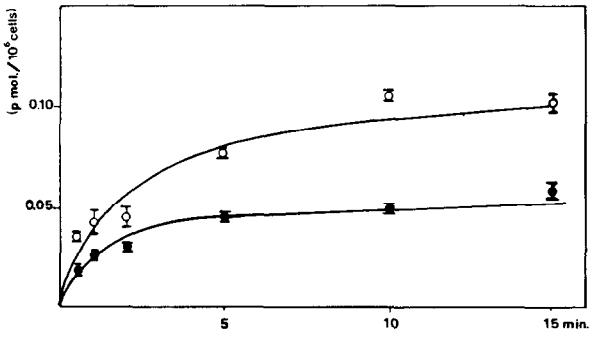

Fig.1. Specific ^{3}H-CB binding as a function of incubation time. ^{3}H-CB used at a concentration of 100 nM. Incubations were done at 22°C as described in Materials and Methods, on fibroblasts from 8-day (•) and 16-day (○) embryos. Abscissa: incubation time in minutes; Ordinate: binding in pmol/10^6 cells.

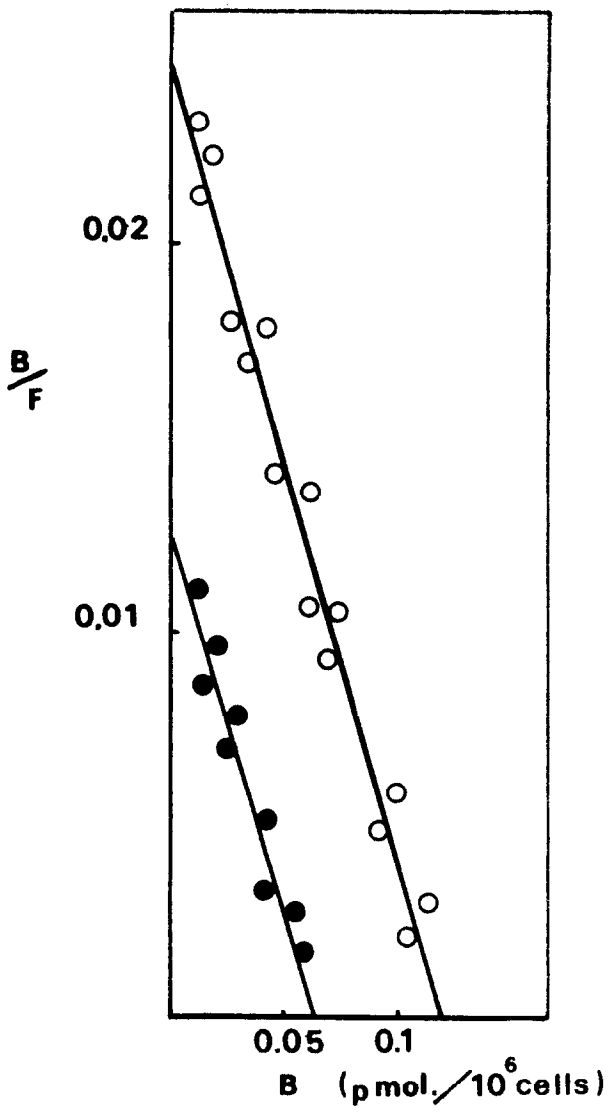

Fig.2. Scatchard plot modified according to Munck [17] of specific ^{3}H-CB binding. The range of ^{3}H-CB concentrations used was 5–2000 nM. Incubations were done at 22°C as described in Materials and Methods, on fibroblasts from 8-day (•) and 16-day (○) embryos. Abscissa: binding (B) in pmol/10^6 cells; Ordinate: ratio of bound (B) to free (F) B/F ^{3}H-CB.

class of CB specific binding sites. An apparent dissociation constant of approximately 50 nM was calculated for the binding sites in both 8- and 16-day cells. There were 2.0 times as many binding sites in the 16-day cells (72 000/cell) as in the 8-day cells (35 000/cell).

3.2. *Effect of various concentrations of 3-O-MeG on ^{3}H-CB binding*

Figure 3 shows the ^{3}H-CB binding in the presence of various concentrations of 3-O-MeG in 8- and 16-day cells. In both cases inhibition was dependent on the 3-O-MeG concentration: 10 to 15 mM 3-O-MeG gave 50% of the maximum inhibition obtained with 50 mM. At a final concentration of 100 mM, 0.128 pmol ^{3}H-CB/10^6 cells were bound in the 16-day cells and in the 8-day cells, 0.065 pmol ^{3}H-CB/10^6 cells. The amount of ^{3}H-CB binding inhibited was double in older than in younger cells, but these values represent the same percentage of inhibition. This suggested that 3-O-MeG competed with CB specific binding sites.

3.3. *Inhibitory effect of CB binding on 3-O-MeG uptake*

The effects of CB on ^{3}H-O-MeG uptake (100 μM, 10 μCi/ml) were measured after a 10 min preincubation at 22°C with CB concentrations rang-

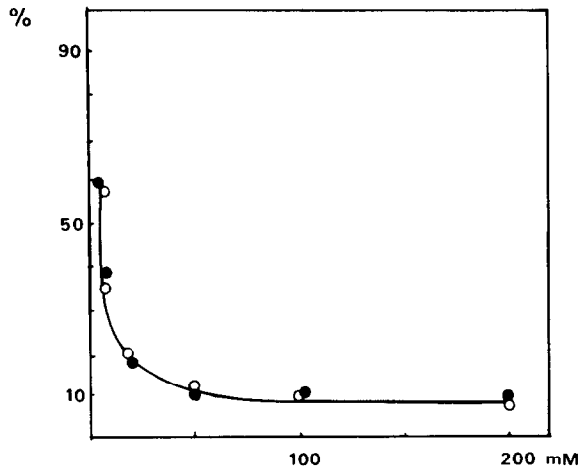

Fig.3. Effect of various concentrations of 3-O-MeG on ^{3}H-CB binding. Cells were simultaneously incubated with 3-O-MeG and ^{3}H-CB. The ^{3}H-CB concentration was 50 mM. The range of 3-O-MeG concentrations used was 5–200 mM. Incubations were done at 22°C as described in Materials and Methods, on fibroblasts from 8-day (•) and 16-day (○) embryos. Abscissa: 3-O-MeG concentrations in mM; Ordinate: ^{3}H-CB binding (binding in the absence of 3-O-MeG minus binding in the presence of 3-O-MeG expressed as % of total binding).

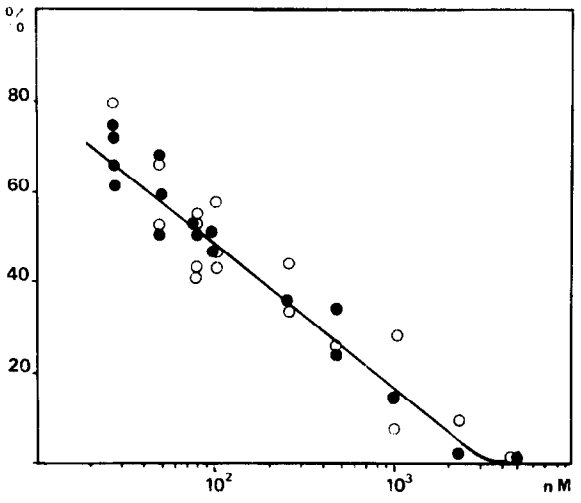

Fig.4. Effect of CB concentrations on 3-O-MeG uptake. Cells were preincubated at 22°C for 10 min with CB concentration ranging from 25 to 5000 nM. 3-O-MeG uptake was then determined, as described in Materials and Methods in fibroblasts from 8-day (•) and 16-day (○) embryos. Abscissa: CB concentration in nM; Ordinate: 3-O-MeG uptake (% of control).

ing from 25 to 5000 nM. In view of the differences in rates of uptake observed between the two stages of development [7], 3-O-MeG uptake was measured after 30 seconds in 8-day cells and after 15 seconds in 16-day cells. As shown in fig.4, 3-O-MeG uptake was more strongly inhibited as the CB concentration increased. At high concentrations CB almost totally inhibited 3-O-MeG uptake in both cell types, and the depression of hexose uptake by the various CB concentrations was similar in the 8- and 16-day cells. The apparent K_i (50% inhibition of 3-O-MeG uptake) was also similar for the two types of cell, i.e., about 60 nM.

4. DISCUSSION

The binding of CB, an effective inhibitor of hexose transport, was studied in order to ascertain whether the increase observed in the rate of hexose uptake in chick embryo fibroblasts [7] could be attributed to an increase in the number of cell membrane carriers between the 8th and 16th day of development, or to a modification of the cell membrane carriers. This latter possibility was excluded since the dissociation constant for the system was similar in both cases, and thus the first hypothesis seemed to be the most probable.

At first it was needed to ensure that CB binding could be used as a probe for the hexose carrier system under our experimental conditions. Although several glucose uptake inhibitors have been described [15], none exhibits the apparent specificity of CB. Phloretin, for example, inhibits glucose uptake in a number of mammalian cells, as well as in chick embryo fibroblasts [7,18], but also inhibits amino acid and nucleoside uptake [19–21]. Our results suggested that 3-O-MeG and CB compete with each other. Salter and Weber [3] obtained similar results with chick embryo fibroblasts but in secondary culture and not during embryo development. The apparent dissociation constant for the CB binding was comparable to the apparent K_i for CB inhibition of 3-O-MeG uptake. It can therefore safely be assumed [3] that the binding sites are associated with the glucose uptake system and that CB binding can be used as a probe for hexose carriers in our cell system.

The analysis of Scatchard plot of CB binding to 8- and 16-day cells according to the modification described by Munck [17] revealed that fibroblasts from 8- and 16-day embryos exhibited only one class of binding sites. These results are at variance with those reported by various authors who found two classes of CB binding sites [3,9–10], but these authors using a classical analysis of the results considered the 'low affinity binding sites' as a true class of binding sites. Under experimental conditions where the 3-O-MeG was not in saturating concentration, we also observed a similar phenomenon. However, we noted that all the 'second class of low affinity' CB binding sites were non-saturable as was observed for steroid hormone receptor sites [17].

The fact that the number of CB binding sites increased 2–3 times in 16-day cells as compared to 8-day cells suggested that the number of glucose carriers increased within embryo development in relation to the rate of 3-O-MeG uptake which increases by a similar factor [7].

These results appeared to be of interest in order to understand certain molecular and biological modifications previously noted between the 8th and the 16th day of development [7,13,22–24].

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