Hexose transport in human myoblasts

Ogubay T. MESMER and Theodore C. Y. LO*

Department of Biochemistry, University of Western Ontario, London, Ontario, Canada N6A 5C1

The present investigation reports on the hexose transport properties of human myoblasts isolated from normal subjects and from patients with Duchenne muscular dystrophy (DMD). Similar to rat myoblast L6, normal human myoblasts possess a high- (HAHT) and a low- (LAHT) affinity hexose transport system. The non-metabolizable hexose analogue, 2-deoxyglucose, is preferentially taken up by HAHT. The transport of this analogue is the rate-limiting step in the uptake process. This human myoblast HAHT is also similar to that of the rat myoblast in its substrate specificity and in response to the energy uncouplers, cytochalasin B and phloretin. The human myoblast LAHT resembles that of rat myoblast in its insensitivity to energy uncouplers, and in its transport affinity and capacity for 3-*O*-methyl-D-glucose. Although DMD myoblasts resemble their normal counterpart in their ability to differentiate, they differ significantly in their hexose transport properties. In addition to HAHT and LAHT present in normal human myoblast, DMD myoblasts contain a super-high-affinity hexose transport system (SHAHT). SHAHT can be detected only at very low substrate concentrations. It differs from HAHT not only in its much higher transport affinity, but also in its response to the traditional hexose transport inhibitors. For example, SHAHT can be activated by cytochalasin B and phlorizin, whereas it is more sensitive to inhibition by phloretin. Unlike HAHT, energy uncouplers are found to be ineffective in inhibiting SHAHT. It should be mentioned that SHAHT cannot be detected in myoblasts isolated from patients with other types of myopathy. The present study serves to demonstrate that more than one hexose transport system is operating in human skeletal muscle cells, as found in other cell types.

INTRODUCTION

The mechanism and regulation of hexose transport have been studied quite extensively in human erythrocytes, adipocytes, fibroblasts, epithelial cells and rat myoblasts (Gliemann & Rees, 1983; Kalckar & Ullrey, 1984; D'Amore & Lo, 1986c; Wheeler & Hinkle, 1985; Simpson & Cushman, 1986). These studies revealed the presence of at least two hexose transport systems in rat myoblasts (D'Amore & Lo, 1986c), chick embryo fibroblasts (Christopher *et al.*, 1976), human skin fibroblasts (Howard *et al.*, 1979), rat adipocytes (Olefsky, 1978; Foley *et al.*, 1980) and mouse 3T3 cells (Colby & Romano, 1975). These systems differ not only in their transport affinity and substrate specificity, but also in their response to various inhibitors. For example, the high-affinity hexose transport (HAHT) system in chick embryo fibroblasts is very sensitive to sulphydryl reagents, whereas the low-affinity (LAHT) system is not (Christopher *et al.*, 1976). Another feature that emerges is that most of the hexose transport systems examined are very sensitive to inhibition by cytochalasin B (D'Amore & Lo, 1986c; Simpson & Cushman, 1986).

Unlike the above-mentioned cell types, not much is known about the hexose transport properties of human muscle cells. Glucose metabolism and utilization in muscles were examined only with muscle fibre bundles (Lundquist & Harris, 1979; Ellis, 1981). Muscle fibres from patients with Duchenne muscular dystrophy

(DMD) were found to take up D-glucose at a much faster rate than those from normal subjects. These DMD muscles were found to convert more glucose to fructose than did muscles from control subjects (Ellis, 1981). Unfortunately, the interpretation of these data is complicated by the general problem that muscle fibres contain different cell types which may not be equally affected (Petell *et al.*, 1984; Chi *et al.*, 1987). While human myoblasts have been isolated and cloned, the hexose transport properties of these cloned cultures have not yet been examined (Blau *et al.*, 1983).

The best-studied muscle hexose transport system is that present in the rat myoblast L6 (Lo & Duronio, 1984a,b; D'Amore & Lo, 1986a,b,c, 1988; D'Amore *et al.*, 1986a,b; Mesmer *et al.*, 1986). Both biochemical and genetic studies revealed the presence of both HAHT and LAHT (D'Amore & Lo, 1986a). D-Glucose and 2-deoxy-D-glucose (dGlc) were transported preferentially by HAHT, whereas 3-*O*-methyl-D-glucose (MeGlc) was transported primarily by LAHT. Similar to those of chick embryo fibroblasts, HAHT was sensitive to low concentrations of sulphydryl reagents, whereas LAHT was not (D'Amore & Lo, 1986b). Both transport systems were inhibited by phloretin but not by phlorizin (D'Amore & Lo, 1986a). The rat myoblast HAHT also differed from LAHT in its dependence on membrane potential (D'Amore & Lo, 1986a). Plasma membrane vesicle studies also showed that D-glucose and dGlc were taken up against a concentration gradient, whereas

Abbreviations used: dGlc, 2-deoxy-D-glucose; MeGlc, 3-*O*-methyl-D-glucose; DMD, Duchenne muscular dystrophy; HAHT, high-affinity hexose transport system; LAHT, low-affinity hexose transport system; SHAHT, super-high-affinity hexose transport system; DNP, 2,4-dinitrophenol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PBS, phosphate-buffered saline; CB, cytochalasin B.

* To whom correspondence and requests for reprints should be addressed.

MeGlc only equilibrated across the plasma membrane (Cheung & Lo, 1984; Mesmer *et al.*, 1986). The K_i values for cytochalasin B (CB) inhibition of HAHT and LAHT differed significantly from one another (D'Amore & Lo, 1986c); CB binding sites associated with the respective hexose transporters were found to have different affinities and substrate specificities (Chen & Lo, 1988). Finally, mutants defective only in HAHT have been isolated. Both transport and CB binding studies with these mutants suggest that the transporters are coded by different genes.

The present investigation reports the hexose transport properties in purified and cloned human myoblasts isolated from normal human subjects and from patients with DMD. This study shows that while two hexose transport systems are also present in normal human myoblasts, DMD myoblasts contain an additional super-high-affinity hexose transport system (SHAHT), which also differs significantly in its response to various hexose transport inhibitors.

MATERIALS AND METHODS

Cell line and culture media

Several clones of human muscle primary cultures were used in this study. Some were generous gifts from Dr. Peter Merrifield, Department of Anatomy, University of Western Ontario and from Dr. Henry Klamut, Hospital For Sick Children, Toronto; the others were isolated in this laboratory. Culture H16D was obtained in April 1976 from a 10-year-old boy with Duchenne muscular dystrophy (H.M., Case # 646008, at University Hospital, University of Virginia). Culture JA was obtained from a boy with DMD at the Hospital For Sick Children, Toronto. The muscle biopsy was taken from the posterior tibialis muscle. This culture was recloned by the dilution technique in our laboratory. Cultures H31, H40 and HD82 were myoblasts isolated from normal male subjects. Culture HS was human myoblasts isolated from a patient with mitochondrial myopathy. Cells were maintained in F-10 medium (Gibco Laboratories) supplemented with 20% (v/v) fetal calf serum, 200 units of penicillin G/ml, 100 units of streptomycin/ml and 50 µg of gentamycin/ml. Transfers were made every week (prior to fusion) by using 0.1% trypsin to dissociate attached cells. Cells were counted with a Coulter counter.

Isolation of human muscle cells

Human myoblasts were isolated essentially as described by Blau (Blau & Webster, 1981; Blau *et al.*, 1983), with slight modifications. Briefly, muscle samples were minced into tiny fragments in ice-cold growth medium, and washed with ice-cold citrate saline. Samples were then trypsinized three times for 20 min with 15–20 ml of 0.05% trypsin in citrate saline at 37 °C with constant shaking. After each trypsin treatment, the supernatant (which contained the dissociated cells) was pooled and cooled at 4 °C; 10% horse serum was added to terminate the protease activity. The dissociated cells were then harvested by centrifugation, and resuspended in growth medium. The growth medium was normal F-10 medium containing 15% fetal calf serum, penicillin G (200 units/ml) and streptomycin sulphate (100 units/ml). Cells were plated on Primaria culture dishes (60 mm × 15 mm) (Falcon). After growing close to confluence, cells were cloned by dilution on 96-well

plates. Wells with a single colony were selected, and recloned before further characterization.

Hexose transport studies

Transport studies were carried out essentially as described earlier (D'Amore & Lo, 1986a). In order to examine the properties of the hexose transport systems independent of the cell density inhibition effect (Chen & Lo, 1989) sub-confluent monolayers were used in these studies. In the determination of transport kinetics, the same amount of radioactive sugar was added to different concentrations of the unlabelled sugar. Cells were grown for 3 days in six-well Costar plates (35 mm × 15 mm) to yield a final cell density of around 3×10^4 cells per well. Medium was aspirated and each well was washed with 8 ml of phosphate buffered saline (PBS; 137 mM-NaCl/2.7 mM-KCl/8.1 mM- Na_2HPO_4 /1.5 mM- KH_2PO_4 /0.9 mM- CaCl_2 , 2H₂O/0.5 mM- MgCl_2). Uptake buffer (PBS containing 1 mg of bovine serum albumin/ml; 900 µl) was added to each well. Transport studies were carried out at 23 °C, and were initiated by the addition of 100 µl of radioactive substrate to the desired final concentration. After appropriate times, uptake was terminated by washing the cells rapidly (less than 15 s) twice with ice-cold PBS or PBS supplemented with 10 mM-mercuric chloride. In all cases, 1-min uptake assays were performed and samples were taken at 15, 30, 45 and 60 s after the addition of the radioactive substrate. Cells were solubilized with 1 ml of 0.1% Triton X-100; 0.8 ml wells on each plate were trypsinized and used to determine the average cell number per well. The initial rates of uptake were calculated from the amount taken up at four time points; zero-time controls and background counts were subtracted from the raw data. All transport studies were carried out in duplicate. Each experiment was repeated at least twice. Figures illustrate the results of representative experiments. Results were found to be very consistent in all cases.

Fate of dGlc after uptake

Transport studies were carried out as described above. Uptake was terminated 1 min after the addition of radioactive dGlc (60 µM) by washing the cells twice with 8 ml of ice-cold PBS containing non-radioactive dGlc (1 mM). The internalized dGlc was then released by treatment with 800 µl of absolute ethanol. The ethanol-soluble materials from six wells were pooled and centrifuged to remove any particulate materials. The phosphorylated and non-phosphorylated dGlc were then separated by h.p.l.c. with a Hewlett-Packard 1084B instrument equipped with a refractive index detector and a Partisil-10 SAX column (Whatman Inc., Clifton, NJ, U.S.A.). The ethanol extracts were evaporated under N₂ at room temperature, and dissolved in 75 µl of water containing unlabelled sugar analogues (100 µl). The compounds were eluted with 35 mM- KH_2PO_4 (pH 4.6) at a flow rate of 1.2 ml/min at 40 °C. Carrier sugars were detected by a refractive index detector and column fractions (360 µl) were collected for liquid scintillation counting. Under these conditions, the phosphorylated and non-phosphorylated sugars were well separated.

Determination of intracellular space

The intracellular space of both normal and DMD myoblasts was determined as described previously (Chen & Lo, 1988) with some modifications. Cells were

incubated with 0.1 mM radioactive MeGlc under conditions similar to those used in transport studies. The amounts of MeGlc internalized at different times were determined. The amount of MeGlc non-specifically bound or trapped by the cells was determined by pretreatment of cells with 10 mM-mercuric chloride; the latter was also included in the uptake buffer. At equilibrium, the amounts of MeGlc present in normal and H16D myoblasts were 12.04 and 11.86 pmol/ 10^5 cells respectively. This indicated that both normal and DMD myoblasts were quite similar in their intracellular volume (1.20 pl/cell). It is interesting to note that this value is quite similar to that of rat myoblast (Chen & Lo, 1988).

Materials

2-Deoxy-D-[G- 3 H]glucose (8.3 Ci/mmol) and 3-*O*-[methyl- 3 H]methyl-D-glucose (60–90 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). HP/b scintillation fluid was from Beckman Instruments (Irvine, CA, U.S.A.). All other chemicals were obtained from commercial sources and were of the highest available purity.

RESULTS

Properties of human myoblasts

Unlike rat myoblast L6, relatively little is known about the properties of human myoblasts. Since the present study involves characterization of the hexose transport system in muscle cells from both normal human subjects and from patients with DMD, a brief description of their properties is justified. Fig. 1 shows that normal human myoblasts (H31) and one of the DMD myoblasts (JA) are relatively small, refractive and triangular cells; whereas the other DMD myoblasts (H16D) are large, flat, distended and pleomorphic cells. Consequently, one cannot determine the nature of the myoblasts based on their morphology. A determination of the intracellular space revealed that both normal and DMD myoblasts were quite similar in their intracellular volume (around 1.20 pl/cell). The protein contents of normal and DMD myoblasts were 14.7 and 12 μ g/ 10^5 cells, respectively; thus they were also similar in their protein contents. It is interesting to note that the protein content of rat myoblast L6 is significantly higher (55 μ g/ 10^5 cells) (Lo & Duronio, 1984a). Both normal and DMD myoblasts were also found to retain the ability to form multinucleated myotubes (Fig. 1). Myotube formation occurred even when the cells were not grown to complete confluence. Similar observations were also made by Blau (Blau & Webster, 1981; Blau *et al.*, 1983) and Jasmin (Jasmin *et al.*, 1984). It should be noted that both undifferentiated human myoblasts and human myotubes are morphologically distinct from those of rat muscle cells (Lo & Duronio, 1984a; D'Amore & Lo, 1988).

As with rat myoblast L6 (D'Amore & Lo, 1988), normal human myoblasts (H31) had an initial doubling time of about 24 h. On the other hand, the DMD myoblast (H16D) had an initial doubling time of about 33 h. The growth rates of both cell lines slowed down considerably after day 5, presumably due to contact inhibition and the gradual depletion of nutrients. These primary human muscle cells exhibited a much higher degree of contact inhibition than the permanent rat myoblast L6.

Hexose transport by human myoblasts

While D-glucose and D-galactose are the natural substrates of the hexose transport system, dGlc is generally used as the non-metabolizable sugar analogue in hexose transport studies (D'Amore & Lo, 1986a). Once internalized, it is phosphorylated by hexokinase to form dGlc 6-*P*, which is not metabolized further. In an initial screening study, the ability of various human myoblasts to take up dGlc was tested. Myoblasts from 'normal' human subjects (H31 and H40) were found to take up 5 μ M-dGlc at a rate of 1.4 pmol/min per 10^5 cells (Fig. 2). On the other hand, the DMD myoblasts (H16D) exhibited a much higher rate of dGlc uptake (2.3 pmol/ 10^5 cells). It should be noted that myoblasts from a patient with mitochondrial myopathy (HS) exhibited the 'normal' rate of uptake (Fig. 2).

If transport was the rate-limiting step, then all of the internalized dGlc should be phosphorylated by hexokinase; conversely, if phosphorylation was the rate-limiting step, then some of the internalized dGlc should remain in the unphosphorylated form. Analysis of the dGlc internalized by H31 and H16D revealed that over 95% existed in the phosphorylated form. Thus, similar to its counterpart in rat myoblasts (D'Amore & Lo, 1986a), transport is the rate-limiting step in the uptake of dGlc by both normal and DMD myoblasts.

Hexose transport kinetics

Increase in the initial rate of transport may be due to changes in transport capacity and/or transport affinity. This can be easily determined by kinetic analyses. Using dGlc concentrations ranging from 5 to 200 μ M, only one hexose transport system could be detected with the normal myoblast (H31); the average K_m and V_{max} values of this system were 140 ± 1 μ M and 66.85 ± 0.15 pmol/min per 10^5 cells respectively (HAHT; Fig. 3a). In addition to this HAHT, a higher affinity hexose transport system (SHAHT) could also be detected in myoblasts from DMD patients (H16D and JA). Their average K_m and V_{max} values were 18.5 ± 2.5 μ M and 14.5 ± 0.15 pmol/min per 10^5 cells respectively (Fig. 3a). This SHAHT was discernible only at very low substrate concentrations. Similar K_m and V_{max} values were also obtained by analysis of the data by Eadie-Hofstee plot (Fig. 3b). Again, dGlc was found to be taken up by only one transport system in normal myoblasts, whereas it was taken up by two different transport processes in DMD myoblasts. This observation may explain why DMD myoblasts exhibited a much higher dGlc transport activity when the substrate concentration was 5 μ M (Fig. 2). Kinetic studies with the other human muscle cultures (H40, HD82 and HS) revealed the presence of only one dGlc transport system; the average K_m and V_{max} values of which were around 160 ± 3 μ M and 53 ± 0.12 pmol/min per 10^5 cells respectively (Fig. 4).

Both biochemical and genetic analyses of rat myoblasts and other cell types suggest that MeGlc, also a non-metabolizable sugar analogue, is taken up primarily by a LAHT (Olefsky, 1978; D'Amore & Lo, 1986a). Both ionophores and energy uncouplers were found to have no effect on the rat myoblast LAHT (D'Amore & Lo, 1986a). As indicated in Fig. 5, such a low-affinity transport system could also be detected in human myoblasts when MeGlc was used as the transport substrate. It is important to note that both normal and

Fig. 1. Morphology of human myoblasts isolated from normal subject and from patients with DMD

Human myoblasts were grown on 100 mm plates and stained with Giemsa stain. (a) Morphology of normal human myoblast H31; (b) morphology of normal human myoblast H40; (c) morphology of multinucleated myotubes formed by H31; (d) and (e) indicate the various shapes and sizes of DMD myoblasts (H16D and JA) respectively. (f) Myotubes formed by H16D.

DMD myoblasts exhibited similar MeGlc transport kinetics; the K_m and V_{max} values were 3.19 ± 0.11 mM and 312 ± 21 pmol/min per 10^5 cells respectively. This can be taken as an indication that DMD myoblasts are altered specifically in the dGlc transport activity, and not in general membrane permeability. Similar to the rat myoblast LAHT, the uptake of MeGlc by both normal and DMD myoblasts was found to be unaffected by $20 \mu\text{M}$ -carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). At an external concentration of 1 mM-MeGlc, the initial rates of uptake by CCCP-treated H40 and H16D myoblasts were 110 and 109% of the respective control values. As will be seen in the next section, this insensitivity of LAHT to CCCP treatment can be used as a criterion to distinguish LAHT from HAHT.

Response of the dGlc transport systems to energy uncouplers

Treatment of whole cells and plasma membrane vesicles with energy uncouplers revealed that the rat myoblast HAHT might be an active transport process (D'Amore & Lo, 1986a; Mesmer *et al.*, 1986). In order to determine the nature of the two dGlc transport systems, the effect of energy uncouplers on the uptake of $5 \mu\text{M}$ -dGlc by both normal (H31) and DMD (H16D) myoblasts was therefore examined. At this concentration, dGlc should be taken up only by HAHT in normal myoblasts, and predominantly by SHAHT in DMD myoblasts. The involvement of LAHT in the uptake of $5 \mu\text{M}$ -dGlc should be negligible. As shown in Table 1, treatment of normal

Fig. 2. Uptake of dGlc by normal and dystrophic human myoblasts

Uptake studies were performed as described in the Materials and methods section. The concentration of dGlc used was $5 \mu\text{M}$, and its specific activity was $2.6 \text{ pmol}/1000 \text{ d.p.m.}$ $\square$ and $\triangle$, Uptake by normal human myoblasts H31 and H40 respectively; $\circ$ and $\bullet$, uptake by myoblasts from patients with DMD (H16D) and mitochondrial myopathy (HS) respectively.

Fig. 3. Hexose transport properties of normal and DMD human myoblasts

Transport studies were carried out as described in the text. The amount of dGlc taken up at 23°C in 15, 30, 45 and 60 s was used to determine the initial rate of uptake (v). The velocity (v) is expressed as nmol of 2-deoxyglucose taken up/min per 10^5 cells. S refers to the concentrations of dGlc (in mmol per litre) used. An equal number of counts was added to each dGlc concentration. $\blacktriangle$, $\triangle$ and $\bullet$, Uptake by the normal human myoblast, (H31) and DMD myoblasts H16D and JA respectively. Analysis of data by Lineweaver-Burk plot; (b) analysis of the same data by Eadie-Hofstee plot.

Fig. 4. Hexose transport kinetic properties of myoblasts from other normal human subjects and from a patient with mitochondrial myopathy

Experiments were carried out as described in the legend to Fig. 3. $\circ$ and $\bullet$, Uptake by the normal human myoblasts H40 and HD82; $\triangle$, uptake by myoblast HS, which was isolated from a patient with mitochondrial myopathy.

myoblasts with $20 \mu\text{M}$ -CCCP resulted in 49% inhibition of dGlc uptake; similar inhibition was also observed with another energy uncoupler, 2,4-dinitrophenol (DNP). Thus, similar to the rat myoblast HAHT (D'Amore & Lo, 1986a), the functioning of the human myoblast HAHT can be inhibited by energy uncouplers. Unlike the normal myoblasts, the uptake of $5 \mu\text{M}$ -dGlc by DMD myoblasts was not significantly affected by either energy uncoupler. This suggests that SHAHT may not be sensitive to the energy uncouplers. If this were the case, then a calculation based on the inhibition of CCCP on the uptake of $5 \mu\text{M}$ -dGlc by normal and DMD myoblasts revealed that the HAHT activity operating in DMD myoblasts was around $0.45 \text{ pmol}/\text{min}$ per 10^5 cells. It follows from this that the activity of SHAHT should be around $1.85 \text{ pmol}/\text{min}$ per 10^5 cells.

In order to explore further the effect of CCCP on SHAHT, the effect of $20 \mu\text{M}$ -CCCP on the transport kinetics of H16D was examined. As shown in Fig. 6(a), the K_m and V_{max} values of SHAHT of the CCCP-treated

Fig. 5. Uptake of MeGlc by normal and DMD human myoblasts

Transport studies were carried out as described in the legend to Fig. 3, except that MeGlc was used as the transport substrate. $\blacktriangle$ and $\bullet$, Uptake by clones H40 (normal) and JA (DMD) respectively.

Table 1. Effects of various hexose transport inhibitors on dGlc transport

Transport studies were carried out as indicated in the text. Inhibitors were added at time zero. Samples were taken at 15, 30, 45 and 60 s. The specific activity of the 5 μ M-dGlc used was 2.5 pmol/1000 d.p.m. Values in parentheses indicate hexose transport as a percentage of control values.

Inhibitors	Hexose transport (pmol/min per 10 ⁵ cells)			
	Normal myoblasts (H31)	DMD myoblasts (H16D)	Calculated levels in DMD myoblasts	
			HAHT*	SHAHT†
Control	1.40 (100%)	2.30	0.40	1.90 (100%)
Energy uncouplers:				
CCCP (20 μ M)	0.72 (51%)	2.07	0.20	1.87 (98%)
DNP (500 μ M)	0.51 (36%)	1.86	0.14	1.72 (91%)
Hexose transport inhibitors:				
Phloretin				
3 μ M	1.28 (91%)	0.68	0.36	0.32 (17%)
5 μ M	1.44 (102%)	0.75	0.41	0.34 (18%)
15 μ M	0.75 (54%)	0.55	0.22	0.33 (17%)
Phlorizin				
10 μ M	1.33 (95%)	2.85	0.38	2.47 (130%)
20 μ M	1.23 (87%)	2.93	0.35	2.58 (136%)
Cytochalasin B				
3 μ M	1.07 (76%)	2.56	0.30	2.26 (119%)
5 μ M	0.42 (30%)	3.36	0.12	3.24 (171%)

* The control value is calculated on the basis that 17.4% of the dGlc transport activity of DMD myoblasts (at an external concentration of 5 μ M) is due to HAHT. The activities in the presence of various sugars are based on the percentage inhibition as determined for normal myoblasts.

† The control value is calculated on the basis that 82.6% of the dGlc transport activity of DMD myoblasts (at an external concentration of 5 μ M) is due to SHAHT. The activities in the presence of various sugars are determined by the difference between total and HAHT transport in DMD myoblasts.

DMD myoblasts were not significantly different from those of the untreated cells. The $V_{\max}$ value of the treated cells was about 16% lower than that of untreated cells. A similar observation was also made when clone JA was treated with 20 μ M-CCCP (Fig. 6b). Thus, in agreement with data presented in Table 1, the functioning of SHAHT is not affected by energy uncouplers such as CCCP.

Effect of specific hexose transport inhibitors on the two dGlc transport systems

The difference between HAHT and SHAHT was further explored using specific hexose transport inhibitors. Phloretin and phlorizin have been shown to exert differential effects on specific hexose transporters (Silverman, 1976; Moran *et al.* 1983; Silverman & Speight, 1986). While phloretin inhibits both HAHT and LAHT in rat myoblast, phlorizin has virtually no effect on these two systems (D'Amore & Lo, 1986a). As shown in Table 1, the rate of uptake of 5 μ M-dGlc by normal human myoblasts was around 1.4 pmol per min/10⁵ cells, and this activity was not altered by treatment with 3 or 5 μ M-phloretin; significant inhibition (46%) could be observed when the phloretin concentration was raised to 15 μ M. Quite a different picture was observed when DMD myoblasts were used in the transport studies. Phloretin (3 or 5 μ M) was found to reduce dGlc transport activity from 2.3 to around 0.72 pmol/min per 10⁵ cells.

Maximal inhibition (but not complete abolishment) of SHAHT was probably observed at 3 μ M-phloretin, as 15 μ M-phloretin could only bring about 7% further reduction of transport activity. This further reduction could be due to the inhibition of HAHT operating at 5 μ M-dGlc. In view of the differential effect of 3 μ M-phloretin on HAHT and SHAHT, this finding suggests that the level of HAHT in DMD myoblasts must be considerably lower than that in normal myoblasts. Based on the above assumption, the activity of SHAHT can then be calculated by a simple algebraic calculation to be around 1.94 pmol/min per 10⁵ cells. This value is similar to the level observed when cells were treated with CCCP. Thus, in the uptake of 5 μ M-dGlc by DMD myoblasts, the average SHAHT activity can be calculated to be 1.90 (average of 1.94 and 1.87) pmol/min per 10⁵ cells. Since the total dGlc transport activity is 2.3 pmol/10⁵ cells, the HAHT activity can be calculated to be 0.4 pmol/min per 10⁵ cells. Based on these figures, it may be surmised that 1.9/2.3 (82.6%) and 0.4/2.3 (17.4%) of the dGlc transport activity in DMD myoblasts is due to SHAHT and HAHT respectively when the external dGlc concentration is 5 μ M (Table 1).

Unlike phloretin, phlorizin had little or no inhibitory effect on human HAHT (Table 1). On the other hand, activation of dGlc uptake was observed when DMD myoblasts were treated with phlorizin. The calculated level of SHAHT in phlorizin-treated cells were more than

Fig. 6. The effect of CCCP on hexose transport by DMD human myoblasts

Transport kinetic studies were carried out essentially as described in the legend to Fig. 3. CCCP was added at zero time; the final concentration of CCCP was 20 μM . (a) Lineweaver-Burk plot of dGlc uptake by H16D cells ($\circ$) and CCCP-treated H16D cells ($\bullet$). (b) Lineweaver-Burk plot of dGlc uptake by JA cells ($\triangle$) and CCCP-treated JA cells ($\blacktriangle$).

33% higher than that in the control cells. Again, this observation serves to illustrate the differences between HAHT and SHAHT.

CB is a potent inhibitor for most of the hexose transport systems tested (Simpson & Cushman, 1986). Treatment of normal human myoblasts with 3 and 5 μM -CB resulted in 24 and 70% loss in transport activity respectively (Table 1). Thus, the human myoblast HAHT is similar to its counterpart in rat myoblast in its response to CB (D'Amore & Lo, 1986c). In sharp contrast with the normal myoblast, treatment of DMD myoblasts with 3 and 5 μM CB was found to activate dGlc uptake by 11 and 46% respectively (Table 1). The calculated levels of SHAHT in cells treated with 3 and 5 μM CB were about 19 and 71% higher than that in control cells. Thus while both transport systems can be modulated by CB, their responses are quite different.

Response to various sugar analogues

In an attempt to further characterize the two dGlc transport systems, the effect of various sugar analogues was tested. In view of the difference in transport affinities, two different concentrations of dGlc were used. As mentioned earlier, 5 μM -dGlc should be taken up by

Fig. 7. The effect of D-glucose on dGlc uptake by DMD myoblast clone JA

Transport studies were carried out as described in the text. D-Glucose was added at the same time as the radioactive dGlc. $\circ$ denotes the effect of D-glucose on the uptake of 5 μM -dGlc by clone JA. The control value was 2.64 pmol/min per 10^5 cells; this was taken as 100% of the activity. $\bullet$, Effect of D-glucose on the uptake of 25 μM -dGlc by clone JA. The control value was 1 pmol/min per 10^5 cells.

HAHT in normal myoblasts, and predominantly by SHAHT in DMD myoblasts. On the other hand, 25 μM -dGlc should be taken up by HAHT in both types of cells (Fig. 3). As indicated in Table 2, while L-glucose had no effect, D-glucose, D-galactose and MeGlc inhibited 25 μM -dGlc uptake in both normal and DMD myoblasts to similar extent. This is probably because only HAHT was operating under these conditions in both cell types. A similar extent of inhibition was observed when the effect of various sugars was tested on the uptake of 5 μM -dGlc by H31. Again, this indicates that only HAHT is operating in normal myoblasts. The presence of SHAHT in DMD myoblasts was apparent when the effect of various sugar analogues was tested on the uptake of 5 μM -dGlc by H16D. D-Galactose and MeGlc seem to be more potent inhibitors than D-glucose in this transport process (Table 2).

If the transporter for SHAHT has the same affinity for D-glucose as that for HAHT, then one would expect that D-glucose should inhibit the uptake of 5 μM -dGlc to a larger extent than that for 25 μM -dGlc in DMD myoblasts. However, data presented in Table 2 show that this is not the case. This finding was confirmed by studies carried out with a range of D-glucose concentrations (0.4–1.6 mM) (Fig. 7). While D-glucose can inhibit the uptake of both 5 and 25 μM -dGlc, the uptake of dGlc by HAHT is consistently inhibited to a much larger extent by D-glucose.

DISCUSSION

The regulation and properties of the hexose transport systems in rat myoblast have been examined through a combination of biochemical and genetic approaches (Lo & Duronio, 1984a,b; D'Amore & Lo, 1986a,b,c; D'Amore *et al.*, 1986a,b; Mesmer *et al.*, 1986; Chen & Lo, 1988). Although glucose metabolism in muscle fibres from both normal and dystrophic human subjects has been investigated (Lundquist & Harris, 1979; Ellis, 1981),

Table 2. Effects of various sugars on dGlc uptake by normal and DMD human myoblasts

Transport studies were carried out as indicated in the text. Non-radioactive sugars (0.6 mM) were added at time zero. Samples were taken at 15, 30, 45 and 60 s. Values in parentheses indicate hexose transport as a percentage of control values.

Sugar analogues	Hexose transport (pmol/min per 10 ⁵ cells)			
	Normal myo- blasts (H31)	DMD myoblasts (H16D)	Calculated levels in DMD myoblasts	
			HAHT*	SHAHT†
(a) Uptake of 5 μM-dGlc				
Control	1.28 (100 %)	2.46	0.43	2.03 (100 %)
L-Glucose	1.28 (100 %)	2.50	0.43	2.07 (102 %)
D-Glucose	0.72 (56 %)	1.76	0.31	1.45 (71 %)
D-Galactose	0.93 (73 %)	1.25	0.31	0.94 (46 %)
MeGlc	0.99 (77 %)	1.49	0.33	1.16 (57 %)
(b) Uptake of 25 μM-dGlc				
Control	10.7 (100 %)	10.0 (100 %)		
D-Glucose	4.3 (40 %)	5.7 (57 %)		
D-Galactose	8.0 (75 %)	7.9 (79 %)		
MeGlc	8.3 (78 %)	8.5 (85 %)		

* The control value is calculated on the basis that 17.4% of the dGlc transport activity of DMD myoblasts (at an external concentration of 5 μ M) is due to HAHT. The activities in the presence of various sugars are based on the percentage inhibition as determined in normal myoblasts.

† The control value is calculated on the basis that 82.6% of the dGlc transport activity of DMD myoblasts (at an external concentration of 5 μ M) is due to SHAHT. The activities in the presence of various sugars are determined by the difference between total and HAHT transport in DMD myoblasts.

hexose transport properties in isolated muscle cells have not been examined. The present investigation reports on the hexose transport properties in cloned human myoblasts. Myoblasts from normal human subjects are morphologically distinct from the rat myoblast L6 (Fig. 1). These primary human muscle cultures exhibit a much higher degree of contact inhibition than the permanent rat myoblast. Although both human and rat myoblasts are capable of undergoing myogenesis, the myotubes formed are also morphologically distinct from each other. Under our growth conditions, normal human myoblast has a doubling time of 24 h. This is similar to that reported by Delaporte *et al.* (1984), in contrast with findings by Blau *et al.* (1983). This is probably due to clonal differences and the growth conditions used.

The hexose transport systems in rat and human myoblasts share a number of common features. (1) They consist basically of both HAHT and LAHT, which are responsible for the uptake of dGlc and MeGlc respectively (Figs. 3, 4 and 5; D'Amore & Lo, 1986a). (2) Transport is the rate-limiting step in the uptake of dGlc. We have shown in this study and previously (D'Amore & Lo, 1986a) that essentially all the internalized dGlc is phosphorylated (D'Amore & Lo, 1986a). (3) Both human and rat HAHT are also similar in their sensitivity to energy uncouplers such as CCCP or DNP (Table 1; D'Amore & Lo, 1986a). On the other hand, CCCP was found to have no effect on either rat or human LAHT. (4) Both rat and human HAHT are also similar in their responses to phloretin, phlorizin, cytochalasin B and various sugar analogues (Tables 1 and 2). The major difference between the human and rat HAHT concerns the kinetic properties of these transport processes. While the rat HAHT has K_m and V_{max} values of 600 μ M

and 250 pmol/min per 10⁵ cells respectively (D'Amore & Lo, 1986a), the corresponding values for the human system are $140 \pm 1 \mu$ M and 66.85 ± 0.15 pmol/min per 10⁵ cells respectively (Fig. 3a).

Data presented in this investigation indicate that the human HAHT differs from the human LAHT not only in its transport affinity and substrate specificity, but also in its response to energy uncouplers, such as CCCP. The finding that at least two hexose transport systems are present in human myoblasts is actually not surprising. Using cDNA probes, at least two glucose transport genes have been identified in mammalian tissues (Wang, 1987; Fukumoto *et al.*, 1988; Sarkar *et al.*, 1988). One of them has been located at human chromosome 3q26.1-->q26.3; whereas the other has been mapped at human chromosome 1p35-->p31.3. The distribution and level of the mRNA for these two genes also vary significantly from tissue to tissue. It was therefore suggested that glucose transport by mammalian tissues may be mediated by a family of related proteins.

A comparison between the normal and DMD myoblasts also reveals some very interesting findings. Fig. 1 shows that DMD myoblasts differ considerably in their morphology; while clone H16D myoblasts are large, flat, distended and pleomorphic cells, clone JA is similar to normal myoblasts in having relatively uniform shape and size. Thus the morphology of myoblasts cannot be used as a criterion to identify DMD myoblasts. It should be noted that DMD myoblasts have a slightly longer doubling time (33 h) than normal myoblasts. Despite these differences, DMD myoblasts retain the ability to differentiate. Similar observations were also made by other workers (Blau *et al.* 1983; Jasmin *et al.* 1984). DMD myoblasts also differ from normal and other

myopathic myoblasts in having an additional SHAHT, which is detectable only at very low substrate concentrations. This transport process differs from HAHT in a number of aspects. (1) Its K_m and V_{max} values are $18.5 \pm 2.5 \mu M$ and $14.15 \pm 0.15 \text{ pmol/min per } 10^5 \text{ cells}$ respectively; these are considerably lower than those of HAHT (Fig. 3). (2) While both energy uncouplers, DNP and CCCP, can inhibit HAHT, they have no effect on SHAHT (Table 1 and Fig. 6). (3) SHAHT also differs from HAHT in its response to various inhibitors (Table 1). Although $5 \mu M$ -phloretin can reduce the activity of SHAHT dramatically, it has no effect on HAHT. On the other hand, whereas phlorizin has hardly any effect on HAHT, it is found to activate SHAHT. Finally, although $5 \mu M$ -CB can bring about 70% inhibition of HAHT activity, it is found to activate SHAHT by 71%. (4) Both transport processes seem to differ slightly in their substrate specificity. D-Glucose seems to inhibit the uptake of $5 \mu M$ -dGlc by SHAHT to a lesser extent than that by HAHT (Fig. 7). On the other hand, dGlc uptake by SHAHT seems to be inhibited to a slightly larger extent by D-galactose and MeGlc (Table 2). It should be noted that SHAHT cannot be detected in human myoblasts isolated from normal subjects or from patients with mitochondrial myopathy, myotonic dystrophy or metabolic myopathy (Figs. 3 and 4; O. T. Mesmer & T. C. Y. Lo, unpublished work).

The finding that DMD myoblasts exhibit greatly elevated hexose transport activity compared with normal ones is in agreement with previous work with muscle fibres. Studies with muscle fibres from both normal and DMD patients revealed that DMD muscles exhibited much higher rate of glucose utilization (the amount of glucose taken up was about 5–6-fold higher than that of normal cells) (Lundquist & Harris, 1979; Ellis, 1981). Since muscle samples contain significant amounts of fibroblasts and adipocytes, it is difficult to define the type of cells responsible for the increased hexose uptake. Our present study using purified myoblasts provides a definitive indication of the altered hexose transport properties of muscle cells from DMD patients.

Recent studies on the identification of the DMD gene suggest that various biochemical alterations are observed only when the DMD gene has been deleted or translocated (Burghes *et al.*, 1987; Koenig *et al.*, 1987; Lev *et al.*, 1987; Monaco & Kunkel, 1988). Thus it seems that the expression of various alterations is suppressed by the normal DMD gene product (dystrophin) (Hoffman *et al.*, 1987; Bonilla *et al.*, 1988). It is at present not certain whether the expression of the additional SHAHT is a direct or indirect consequence due to the absence of the normal dystrophin. In view of the observation that the DMD transcript cannot be detected in primary human myoblasts prior to the formation of myotubes (Lev *et al.*, 1987; Nudel *et al.*, 1988), it may be surmised that there is no correlation between the absence of dystrophin and SHAHT, as the latter is not observed in normal myoblasts. The fact that SHAHT is only observed in DMD myoblasts suggests that this transport system may be another manifestation of the alterations in DMD myoblasts. On the other hand, it is also possible that the elevated proteolytic activity observed in DMD cells may lead to the activation of a normally inactive hexose transport system (Gopalan *et al.*, 1986; Reddy *et al.*, 1986). Limited proteolytic cleavage has previously been shown to activate the hexose transport systems in rat

myoblast (Lo & Duronio, 1984b; D'Amore *et al.*, 1986a). The finding that LAHT and HAHT are not altered indicates that the observed increase in transport activity is due to specific changes, and not due to alterations in general membrane permeability.

In summary, we have characterized the properties of the hexose transport systems in both normal and DMD human myoblasts. The present study shows that at least two different hexose transport systems are present in human muscle cells. While the hexose transport systems in both rat and human myoblasts are quite similar, the DMD human myoblasts differ from their normal counterpart in the expression of an additional SHAHT.

This investigation was supported by operating grants from the Medical Research Council of Canada to T. C. Y. L.

REFERENCES

- Blau, H. M. & Webster, C. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 5623–5627
- Blau, H. M., Webster, C. & Pavlath, G. K. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4856–4860
- Bonilla, E., Samitt, C. E., Miranda, A. F., Hays, A. P., Salvati, G., Di Mauro, S., Kunkel, L. M., Hoffman, E. P. & Rowland, L. P. (1988) *Cell* **54**, 447–452
- Burghes, A. H. M., Logan, C., Hu, X., Belfall, B., Worton, R. G. & Ray, P. N. (1987) *Nature (London)* **328**, 434–437
- Chen, S. R. & Lo, T. C. Y. (1988) *Biochem. J.* **251**, 63–72
- Chen, S. R. & Lo, T. C. Y. (1989) *J. Cell. Physiol.* **138**, 338–348
- Cheung, M. O. & Lo, T. C. Y. (1984) *Can. J. Biochem. Cell Biol.* **62**, 1217–1227
- Chi, M. M. Y., Hintz, C. S., McKee, D., Felder, S., Grant, N., Kaiser, K. K. & Lowry, O. H. (1987) *Metabolism* **36**, 761–767
- Christopher, C. W., Kohlbacher, M. S. & Amos, H. (1976) *Biochem. J.* **158**, 439–450
- Colby, C. & Romano, A. H. (1975) *J. Cell. Physiol.* **85**, 15–24
- D'Amore, T. & Lo, T. C. Y. (1986a) *J. Cell. Physiol.* **127**, 95–105
- D'Amore, T. & Lo, T. C. Y. (1986b) *J. Cell. Physiol.* **127**, 106–113
- D'Amore, T. & Lo, T. C. Y. (1986c) *Biochem. Cell Biol.* **64**, 1081–1091
- D'Amore, T. & Lo, T. C. Y. (1988) *Biochem. J.* **250**, 59–64
- D'Amore, T., Cheung, M. O., Duronio, V. & Lo, T. C. Y. (1986a) *Biochem. J.* **238**, 831–836
- D'Amore, T., Duronio, V., Cheung, M. O. & Lo, T. C. Y. (1986b) *J. Cell. Physiol.* **126**, 29–36
- Delaporte, D., Dehaupas, M. & Fardeau, M. (1984) *J. Neurol. Sci.* **64**, 149–160
- Ellis, D. A. (1981) *Br. Med. Bull.* **36**, 165–171
- Foley, J. E., Foley, R. & Gliemann, J. (1980) *Biochim. Biophys. Acta* **599**, 689–698
- Fukumoto, H., Seino, S., Imura, H., Seino, Y., Eddy, R. L., Fukushima, Y., Byers, M. G., Shows, T. B. & Bell, G. I. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5434–5438
- Gliemann, J. & Rees, W. D. (1983) *Curr. Top. Membr. Transp.* **18**, 339–379
- Gopalan, P., Dufresne, M. J. & Warner, A. H. (1986) *Biochem. Cell Biol.* **64**, 1010–1019
- Hoffman, E. P., Monaco, A. P., Feener, C. E. & Kunkel, L. M. (1987) *Science* **238**, 347–350
- Howard, B. V., Mott, D. M., Fields, R. M. & Bennett, P. H. (1979) *J. Cell. Physiol.* **101**, 129–138

- Jasmin, G., Tautu, C. & Proschek, J. (1984) *Exp. Biol. Med.* **9**, 102–107
- Kalckar, H. M. & Ullrey, D. B. (1984) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **43**, 2242–2245
- Koenig, M., Hoffman, E. P., Bertelson, C. J., Monaco, A. P., Feener, C. & Kunkel, L. M. (1987) *Cell* (Cambridge, Mass.) **50**, 509–517
- Lev, A. A., Feener, C. C., Kunkel, L. M. & Brown, R. H., Jr. (1987) *J. Biol. Chem.* **262**, 15817–15820
- Lo, T. C. Y. & Duronio, V. (1984a) *Can. J. Biochem. Cell. Biol.* **62**, 245–254
- Lo, T. C. Y. & Duronio, V. (1984b) *Can. J. Biochem. Cell. Biol.* **62**, 255–265
- Lundquist, I. & Harris, J. B. (1979) *Eur. J. Pharmacol.* **53**, 365–373
- Mesmer, O., Cheung, M. O., D'Amore, T. & Lo, T. C. Y. (1986) *Biochem. Biophys. Res. Commun.* **141**, 124–130
- Monaco, A. P. & Kunkel, L. M. (1988) *Adv. Human. Genet.* **17**, 61–98
- Moran, A., Turner, R. J. & Handler, J. S. (1983) *J. Biol. Chem.* **258**, 15087–15090
- Nudel, U., Robzyk, K. & Yaffe, D. (1988) *Nature* (London) **331**, 635–638
- Olefsky, J. M. (1978) *Biochem. J.* **172**, 137–145
- Petell, J. M., Marshall, N. A. & Lebherz, H. G. (1984) *Int. J. Biochem.* **16**, 61–67
- Reddy, P. A., Anandavali, T. E. & Anandaraj, M. P. J. S. (1986) *Clin. Chim. Acta* **160**, 281–288
- Sarkar, H. K., Thorens, B., Lodish, H. F. & Kaback, H. R. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5463–5467
- Silverman, M. (1976) *Biochim. Biophys. Acta* **457**, 303–351
- Silverman, M. & Speight, P. (1986) *J. Biol. Chem.* **261**, 13820–13826
- Simpson, I. A. & Cushman, S. W. (1986) *Annu. Rev. Biochem.* **55**, 1058–1089
- Wang, C. (1987) *J. Biol. Chem.* **262**, 15689–15695
- Wheeler, T. J. & Hinkle, P. C. (1985) *Annu. Rev. Physiol.* **47**, 503–517

Received 26 September 1988/6 March 1989; accepted 16 March 1989