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Glucose transporter 3 (GLUT3) protein is present in human myocardium

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Abstract

Glucose and fructose enter mammalian cells via facilitated diffusion, a process regulated by five glucose transporter isoforms (GLUT1–5) at the plasma membrane. The tissue-specific pattern of GLUT isoform expression likely reflects differing needs for glucose transport by various tissues. Myocytes must respond expeditiously to increased metabolic demand. A basal isoform, GLUT1, and the insulin-regulatable glucose transporter, GLUT4, have been demonstrated in human myocytes. GLUT3 has a high affinity for glucose, but its presence in human myocardium has not been clearly established. The purpose of this study was to determine whether GLUT3 protein is present in human cardiac myocytes. We examined rapidly frozen myocardial tissue from the explanted heart of seven patients undergoing cardiac transplantation, from the heart of a young, previously healthy male organ donor, from the heart of a 67-year-old woman without known cardiac disease who had a fatal stroke, and from the heart of six human fetuses. GLUT3 protein was detected by immunoblots and localized by light and electron microscopy immunohistochemistry. The presence of GLUT3 protein was verified in myocardial tissue by both immunoblots and immunohistochemistry. Light and electron microscopy confirmed that GLUT3 was in cardiac myocytes. GLUT3 was also demonstrated as a 48 kDa protein in fetal myocardium, which was present at 10 weeks, increased at 15 weeks, then decreased at 20 weeks of gestation. GLUT3 is present in human adult and fetal myocardium. Human myocardial GLUT3 regulation and its role in myocardial glucose uptake remain to be elucidated.

Keywords: Glucose transporter; Heart muscle; (Human)

1. Introduction

Glucose and fructose transport into mammalian cells is facilitated by five functional GLUT isoforms, GLUT1–5 [1–3]. Although the amino acid sequence similarity among the isoforms is high, isoform expression is tissue- and species-specific [1]. Two GLUT isoforms, GLUT1 and GLUT4, have been demonstrated in myocytes [4–6]. GLUT1 has a low K_m and provides many cells with their basal glucose requirement whereas GLUT4 mediates insulin stimulated glucose transport [7]. The tissue-specific pattern of GLUT expression likely reflects differing needs for glucose transport by various tissues [2].

Myocytes must respond expeditiously to differing needs for glucose transport. Among GLUT isoforms, GLUT3 has the lowest K_m (1.4 mM for 2-deoxy-D-glucose [8]). Whether GLUT3 is present in human myocardium is not clear. One study reported GLUT3 in human myocardium and another did

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not [9,10]. The human GLUT3 (hGLUT3) cDNA was initially cloned using skeletal muscle from a 20–22-week-old fetus [11]. hGLUT3 is found predominantly in neural tissue, placenta and testes [3] and has been described in platelets [12,13]. Because of sequence differences at the carboxy terminus of the human and murine proteins [14], human and murine antibodies which bind to the GLUT3 carboxy terminus do not cross-react [15]. In addition, GLUT3 tissue distribution is different in humans and mice [15]. In humans, hGLUT3 mRNA [11,16] has been found in adult skeletal muscle. Despite the fact that GLUT3 mRNA [17] and protein [18–22] were detected in rat myoblasts and myotubes, GLUT3 mRNA was not present in monkey or rat skeletal muscle [11] and GLUT3 protein was not present in mouse skeletal muscle [15].

The purpose of this study was to determine whether GLUT3 protein is present in human adult and fetal myocardium and to examine whether it is localized to myocytes.

2. Materials and methods

2.1. Patient population

The protocols to obtain tissue were approved by the University of Iowa Human Subjects Committee.

2.1.1. Explanted hearts

Myocardial tissue was obtained as described below from the explanted heart in seven adult cardiac transplant recipients with cardiomyopathy, four with coronary artery disease (ischemic cardiomyopathy) and three without coronary artery disease (dilated cardiomyopathy). Myocardial tissue was similarly obtained from an organ donor, a previously healthy 12-year-old male. Following an accident, he received several hours of intravenous medication for blood pressure support and was subsequently declared brain dead. Hemodynamic instability precluded adequate assessment of his heart for cardiac transplantation. Myocardial tissue was obtained from a 67-year-old woman without known cardiac disease who had a fatal stroke. The heart was preserved on ice for approximately 8 h prior to obtaining tissue samples.

2.1.2. Fetal tissue

Myocardial and skeletal muscle (gastrocnemius) tissue was obtained as described below from six fetuses of 10 to 21 weeks gestational age. Fetal ages were estimated by a combination of gestational dates, an estimate by the examining gynecologist, and foot length [23].

2.2. Acquisition of myocardial tissue

Explanted hearts were obtained by one of the authors (MGM) in the operating room as they were excised. Myocardial tissue samples (at least 1 cm^3) were obtained immediately from the anterior left ventricular wall. Tissue samples from the proximal and distal portion of a given myocardial wall were obtained in all hearts except the 12-year-old organ donor in whom only a mid anterior wall sample was obtained. The tissue was divided into epicardial and endocardial samples, immediately frozen in liquid nitrogen, and stored at −70°C until use. Separate samples for light and electron microscopy were obtained. The fetal tissue was obtained and frozen in liquid nitrogen by one of the authors (MGM) immediately after elective abortion.

2.3. Quantification of GLUT3 protein

2.3.1. Tissue preparation

Using an affinity purified rabbit polyclonal hGLUT3 antibody, R1672, obtained from Dr. Gwyn Gould [24], GLUT3 protein was assessed using a modified, previously published protocol which investigated GLUT1 [25]. The tissue was placed in cold TES buffer (10 mM Tris–HCl (pH 7.4), 250 mM sucrose, 2 mM EDTA, 1.5 mM phenylmethylsulfonylfluoride), which included protease inhibitors, and homogenized with a Tekmar Tissumizer (Cincinnati, OH). Solubilized membrane proteins were prepared by centrifugation in TES buffer. Protein was determined by the method of Bradford [26] using the Bio-Rad protein assay (Hercules, CA).

2.3.2. Immunoblots

Samples were resolved on 10% SDS–polyacrylamide gel electrophoresis, and electrically transferred to HyBond C membrane (Amersham, Arlington Heights, IL). Immunoblots were developed by en-

hanced chemiluminescence (ECL; Amersham). Membranes were blocked by a 10 min incubation in 10% non-fat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T; pH 7.4), rinsed in TBS, incubated with diluted primary antibody (rabbit anti-human GLUT3 (1:500), anti-rat GLUT1 (5 μg/ml), or anti-rat GLUT4 (5 μg/ml) for 1 h at room temperature with agitation, rinsed twice with TBS-T for 10 min each time, incubated for 15 min with horseradish peroxidase-linked secondary antibody anti-rabbit Ig from donkey (1:1000; Amersham), and then rinsed with TBS-T. Membranes were then incubated 1 min with ECL detection solutions 1 and 2, and exposed to autoradiography film (Hyper-film MP, Amersham) for approximately 5 s. The membranes were stained with Ponceau-S to ascertain even protein loading.

2.3.3. Peptide blocking

To investigate specificity of R1672 binding with GLUT3, an immunoblot was incubated with both GLUT3 peptide (50 μg/ml; Alpha Diagnostic, San Antonio, TX) and the primary antibody for 1 h. All other procedures were as described above.

2.3.4. Cross-reactivity with actin

To determine hGLUT3 cross-reactivity with actin, the following amounts of human brain and actin protein were loaded onto two separate gels: 3, 10, 30, 100, 300 and 1000 ng. One immunoblot was incubated with R1672 and the other with a rabbit anti-actin antibody (10 μg/ml; Sigma, St. Louis, MO) for 1 h. All other procedures were as described above.

2.3.5. Materials

The hGLUT3 antibody, R1672, an affinity purified rabbit polyclonal antibody, was kindly provided by Dr. Gwyn Gould [9,24]. This antibody was made against a peptide corresponding to the 14 COOH terminal amino acids of hGLUT3 (NH_2-MNSIEPAKETTNV-COOH). Antibodies to GLUT1 and GLUT4 were purchased from Alpha Diagnostic (San Antonio, TX). Rabbit actin was provided by Dr. Larry Tobacman. Rabbit anti-actin antibody and rabbit serum were obtained from Sigma. The platelet-specific antibody to glycoprotein IIIa, CD61, was obtained from DAKO Corp. (Carpinteria, CA).

2.4. Immunolocalization of GLUT3 protein

2.4.1. Light microscopy

Tissues were fixed in 10% buffered formalin, then embedded in paraffin using routine procedures. Sections (6 μm) were cut and mounted on Fisher Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) and warmed at 45°C until dry. Tissues were deparaffinized and rehydrated using routine procedures. Immunogold labeling was performed as follows (Aurion, Wageningen, Netherlands) [27,28]. Tissues were blocked in 50 mM glycine in phosphate-buffered saline (PBS; pH 7.4) for 30 min at room temperature (RT) then in 5% acetylated bovine serum albumin (BSA-C; Aurion) and 5% normal goat serum (NGS) for 30 min at RT. Two washes followed, 5 min each, in incubation buffer (IB; 0.1% Aurion BSA-C in PBS). Overnight incubation in GLUT3 primary antibody (R1672) diluted 1:500 in IB was carried out at 4°C. Tissues were then washed four

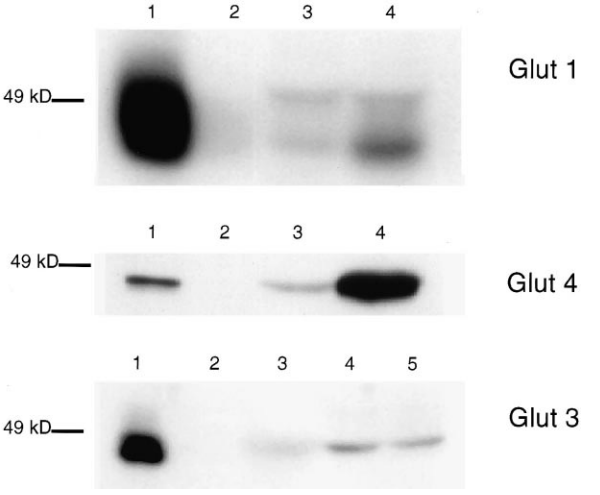

Fig. 1. Western blots from the anterior myocardial wall from a previously normal 12-year-old boy. GLUT1 (top); GLUT4 (middle); hGLUT3 (bottom). Each control lane was loaded with 10 μg of protein and the lanes with myocardium were loaded with 30 μg of protein. Myocardial tissue was obtained from the mid anterior wall and was divided into epicardial and endocardial samples. GLUT1 lanes: 1, human brain; 2, carbonate-treated rat skeletal muscle; 3, mid anterior wall epicardium; 4, mid anterior wall endocardium. GLUT4 lanes: 1, carbonate-treated rat skeletal muscle; 2, human brain; 3, mid anterior wall epicardium; 4, mid anterior wall endocardium. GLUT3 lanes: 1, human brain; 2, carbonate-treated rat skeletal muscle; 3, rabbit actin; 4, mid anterior wall epicardium; 5, mid anterior wall endocardium.

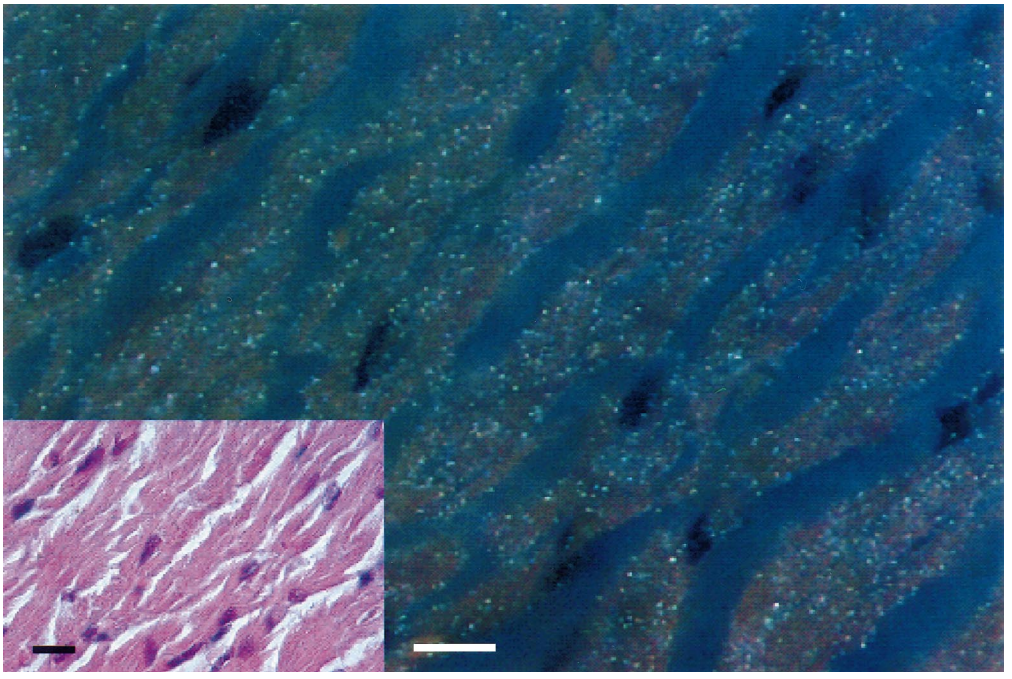

Fig. 2. Immunohistochemical localization of hGLUT3 using light microscopy in the distal anterior wall of a 67-year-old woman without a history of heart disease. Myocytes and nuclei are delineated by hematoxylin–eosin stain (inset). The GLUT3 antibody is conjugated with silver-enhanced gold particles that depolarize and reflect the polarized incident light and show up as bright spots against a dark background. Presence of the bright spots within myocytes provides localization of hGLUT3. Bar = 10 μm.

times for 5 min each in IB. Incubation in a 1:75 dilution of secondary antibody consisting of goat anti-rabbit IgG ultra small gold particles (0.8 nm) in IB for 3 h at RT. Following this, tissues were washed in IB four times for 5 min each, followed by a 5-min wash in PBS, then five washes in deionized water for 2 min each. Post-fixation was in 2.5% glutaraldehyde in PBS for 5 min, and five washes in deionized water for 2 min each followed. Silver enhancement was performed by incubation with Aurion R-Gent for 25 min at RT in the dark. Tissues were rinsed in deionized water, counterstained with hematoxylin–eosin, mounted in permount (Electron Microscopy Sciences, Ft. Washington, PA), and dried on a slide warmer for 24–48 h. Assessment of non-specific staining was accomplished using normal rabbit serum instead of the GLUT3 antibody. Negative control tissues were immunostained as above, but in normal rabbit serum instead of GLUT3 antibody.

Sections were viewed on a light microscope (Leitz Diaplan; Leica, Wetzlar, Germany). Silver-enhanced gold particles were visualized and photographed using an epi-polarization block. The silver-enhanced gold particles depolarize and reflect the polarized incident light and show up as bright spots against a dark background.

2.4.2. Transmission electron microscopy

For histology, tissues from explanted hearts were fixed in 2.5% glutaraldehyde in cacodylate buffer, postfixed in 1.0% buffered osmium tetroxide containing 1.5% potassium ferrocyanide, then washed, dehydrated in acetone and embedded in Spur's resin. Ultrathin sections were stained with uranyl acetate and lead citrate.

For immunohistochemistry, tissues were minced and fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in PBS (0.05 M), overnight at 4°C. Tissues were washed with PBS twice, then rinsed in deionized water. Dehydration was carried out in 70% ethanol (ETOH), two washes for 30 min each. Tissues were incubated in (2:1) 70% ETOH and LR White resin (Polysciences, Warrington, PA). Infiltration with 100% LR White resin was carried out at RT overnight. Two short washes with LR White resin was followed by polymerization using thermal curing at 65°C for 24 h. Sections (100 nm) were

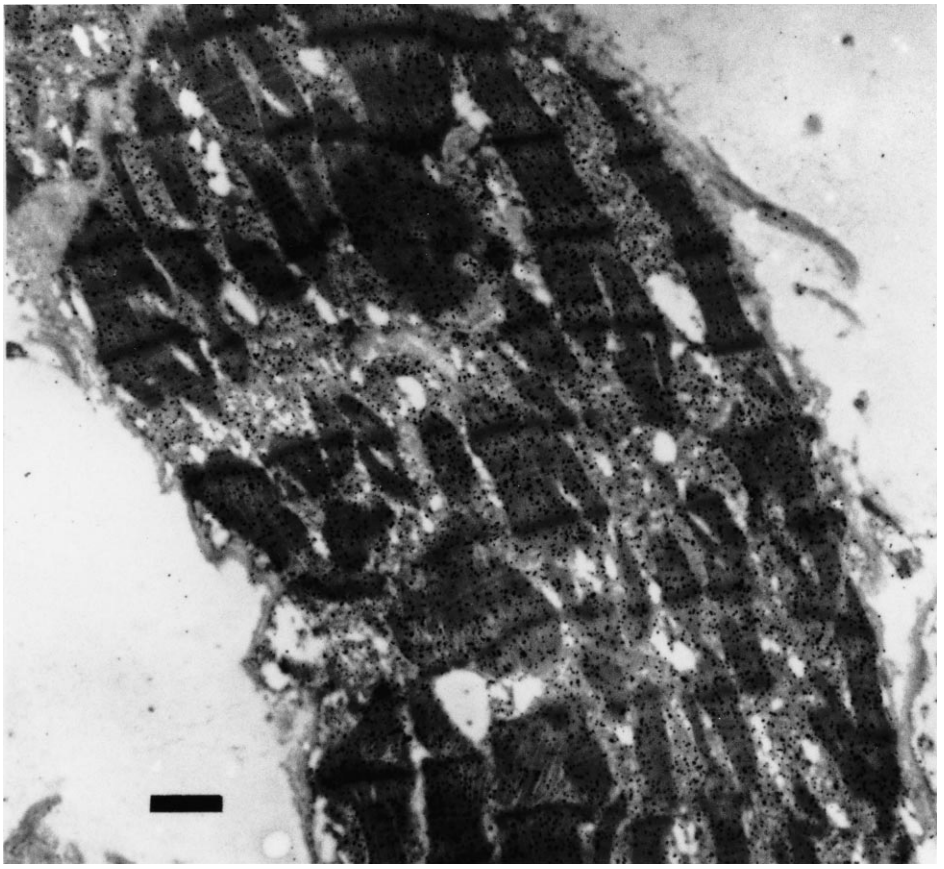

Fig. 3. Immunohistochemical localization of hGLUT3 using electron microscopy in the epicardium of the anterior wall in the woman without a history of cardiac disease. hGLUT3 localization. Ultrasmall gold conjugates are localized in myocytes. Particles are not noted in the electron lucid area between myofibers. Membranes, including mitochondrial membranes, are not readily demonstrated with paraformaldehyde fixation without osmium postfixation. Extremely little non-specific staining is present. Bar = 1 μm.

collected on formvar-coated, 200-mesh nickel grids (Electron Microscopy Sciences). Immunogold labeling was performed as above with the following exceptions. Blocking in 50 mM glycine in PBS was for 15 min. After blocking with 5% BSA and 5% NGS in PBS, three washes in IB were performed. After overnight incubation in GLUT3 primary antibody, six washes in IB were performed. The dilution of the secondary antibody, goat anti-rabbit IgG ultrasmall gold particles (0.8 nm), was 1:40; subsequently six washes in IB and three washes in PBS were performed. After silver enhancement, five rinses in deionized water were performed. Heavy metal staining was performed in 5% uranyl acetate for 6 min followed by three rinses for 1 min each in deionized water. Sections were then counterstained in lead citrate for 8 min and washed with 0.02 N NaOH six times, 1 min each, then washed in deionized water six times, 1 min each. Grids were allowed to air dry. Sections were viewed using a Hitachi H7000 transmission electron microscope (Tokyo, Japan).

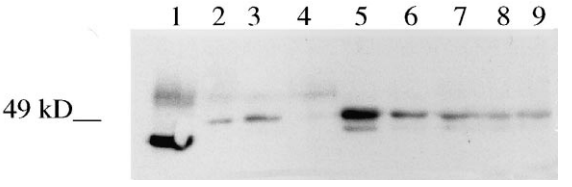

Fig. 4. GLUT3 Western blot using fetal heart. All lanes were loaded with 15 μg of protein. Lanes: 1, human brain; 2, fetal heart at 10 weeks gestation; 3, fetal heart at 15 weeks gestation; 4, fetal heart at 20 weeks gestation; 5, mid anterior wall epicardium from the previously normal subject; 6, mid anterior wall endocardium from the previously normal subject; 7, skeletal muscle at 15 weeks gestation; 8,9, skeletal muscle at 20 weeks gestation.

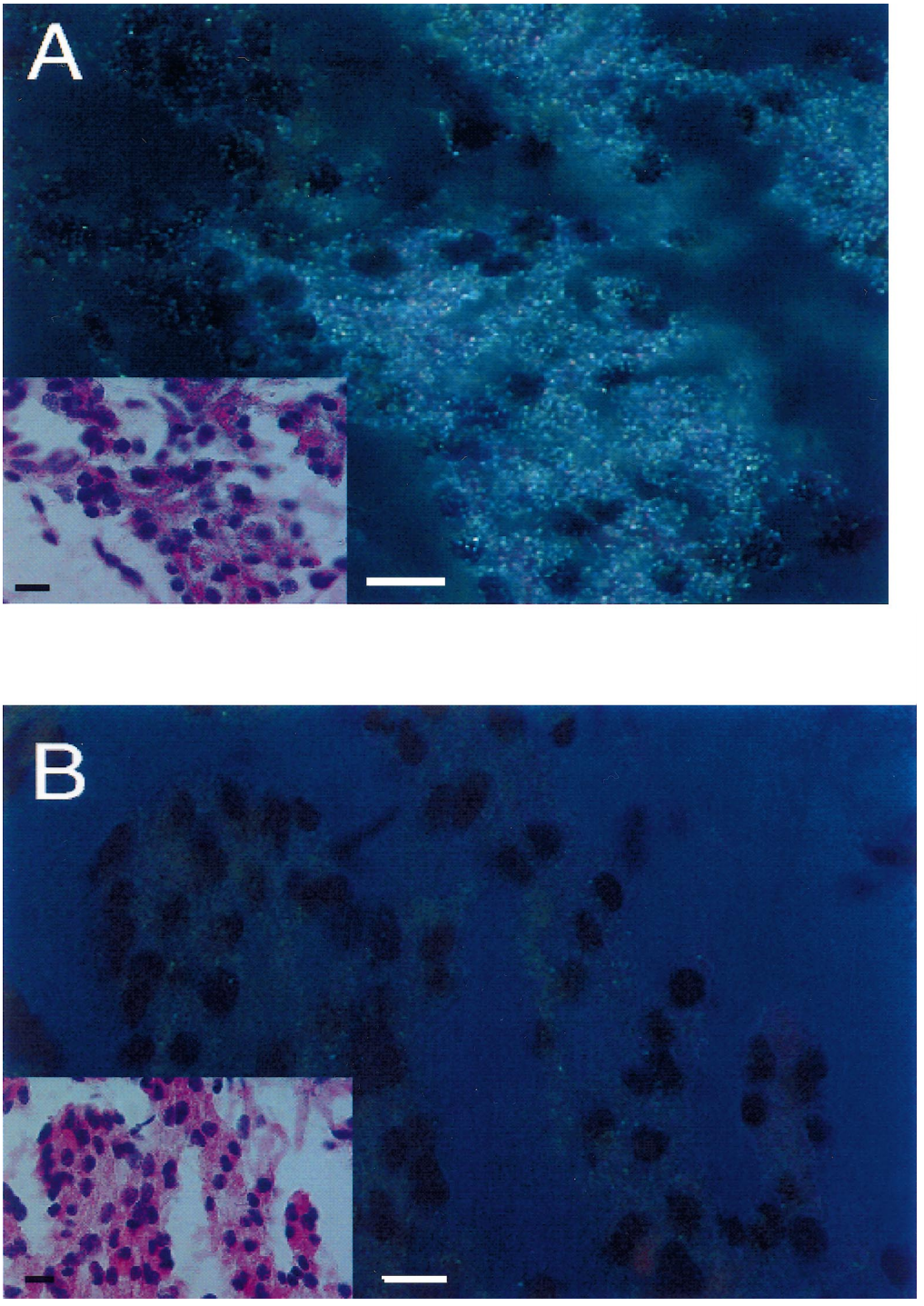

Fig. 5. Immunohistochemical localization of hGLUT3 using light microscopy in fetal myocardium. Myocytes and nuclei are delineated by hematoxylin and eosin stain (insets). Bar = 10 μm. (A) Left ventricular myocardium from a 15-week-old fetus. hGLUT3 staining was intense in myocytes of a 15-week-old fetus. Extremely little non-specific staining is present. (B) Left ventricular myocardium from a 21-week-old fetus. hGLUT3 staining was faint in myocytes of the 21-week-old fetus.

2.4.3. Immunohistochemical identification of platelets

To determine the contribution of GLUT3 from platelets in the myocardial tissue, immunohistochemistry was performed using the platelet-specific antibody to glycoprotein IIIa, CD61 [29,30], and the immunogold technique described above for light and transmission electron microscopy. Platelets were prepared using the following method. Whole human blood was collected in a 7 ml vacutainer tube without any anticoagulants. The blood was allowed to clot on ice for 20 min. The clotted blood was then fixed in 4% paraformaldehyde for electron microscopy or 10% buffered formalin for light microscopy.

3. Results

3.1. Explanted hearts

3.1.1. Western blots

GLUT1 and GLUT4, and GLUT3 were present in myocardium from a previously healthy 12-year-old boy (Fig. 1). In patients with cardiomyopathy, GLUT3 expression appeared to be less than in the previously healthy boy. GLUT1 and GLUT4 expression appeared to be decreased in the endocardium compared with the epicardium, which was more noticeable in the distal wall samples and in patients with ischemic cardiomyopathy (data not shown).

Specificity of the GLUT3 antibody was confirmed by co-incubating human myocardial tissue with rabbit serum, which resulted in no binding on an immunoblot, and with the hGLUT3 peptide which resulted in blocking all GLUT3 antibody binding on an immunoblot and by immunohistochemistry (data not shown). Co-incubation of human myocardial tissue with GLUT3 antibody and actin peptide resulted in decreased actin binding. The higher specificity of R1672 for GLUT3 than actin was confirmed when R1672 detected brain GLUT3 in the 300 ng and 1000 ng protein samples whereas actin was not clearly detectable in these samples. Anti-actin antibody detected actin in the 300 ng and 1000 ng protein samples but did not detect GLUT3 (data not shown).

3.1.2. Immunohistochemistry

After incubation with hGLUT3 antibody, specific reaction product, indicating presence of hGLUT3, was seen over myocytes both by light and electron microscopy (Figs. 2 and 3). No reaction product was observed in the absence of hGLUT3 antibody and in the presence of rabbit serum (data not shown). By electron microscopy, large, electron lucid spaces between myofibers contained very few gold particles. These spaces were filled with mitochondria when conventional electron microscopy fixation and staining techniques were used (i.e., glutaraldehyde with osmium post-fixation).

Platelets were identified in blood using CD61 but were extremely scarce in the myocardial tissue sample using electron microscopy (data not shown).

3.2. Fetal heart and skeletal muscle

3.2.1. Western blots

hGLUT3 was present in fetal heart at 10 weeks of gestation (Fig. 4, lane 2), appeared to increase at 15 weeks (Fig. 4, lane 3), and to decrease at 20 weeks (Fig. 4, lane 4). hGLUT3 was present in fetal skeletal muscle at 15 and 20 weeks of gestation (Fig. 4, lanes 7–9).

3.2.2. Immunohistochemistry

hGLUT3 staining was intense in myocytes of the 15-week-old fetus (Fig. 5A) whereas only faint staining was present in myocytes of the 21-week-old fetus (Fig. 5B).

4. Discussion

We have demonstrated the presence of GLUT3 protein in human adult and fetal myocardium and in fetal skeletal muscle. Immunohistochemistry of adult and fetal myocardium localized hGLUT3 within myocytes.

Previously one group detected hGLUT3 in human myocardium [9] and another did not [10]. One possible explanation for the disparate findings is that the antibodies used for immunoblots were different. The antibodies for human GLUT3 were made using a peptide corresponding to the 14 [9] or 11 [10] COOH-terminal amino acids. The GLUT3 antibody we used does not recognize GLUT1, -2, –4 or -5 proteins [9]. A second possible factor contributing

to the contradictory findings regarding the presence of GLUT3 is the length of time between death and when the tissue was frozen. Haber et al. [10] acquired human heart tissue less than 24 h after death, whereas this information was not provided in the study by Shepherd et al. [9]. In our study, we sought to minimize protein degradation, so tissue was obtained and frozen in the operating room immediately as the heart was explanted. A third possible factor is that hGLUT3 expression is differentially regulated in cardiac disease. Preliminary data from our laboratory suggest that hGLUT3 expression may be less in hearts of patients with cardiomyopathy than in hearts of people without known heart disease.

We demonstrated that the hGLUT3 protein is approximately 48 kDa, as previously described [9,10]. We observed different migration of brain and myocardial hGLUT3 on the polyacrylamide gel, a finding likely due to differential glycosylation. In addition to the different molecular masses on immunoblots of GLUT3 and actin, we demonstrated that the affinity of R1672 was much greater for GLUT3 than for actin. Because platelets contain GLUT3 [12,13], we performed immunohistochemistry, demonstrating that the contribution of GLUT3 from platelets in human myocardium is extremely small. Therefore, our immunohistochemistry data confirm the presence of GLUT3 in human myocardium.

The presence of hGLUT3 in human but not rodent myocardium implies that it may not be possible to use murine models to investigate the role of the different GLUT isoforms on glucose metabolism in human myocardial disease. An animal with myocardial GLUT3 must be identified to conduct such studies.

Thus, human myocardium contains GLUT1, GLUT4, and GLUT3. The relative expression of GLUT isoforms in myocardial health and disease remains to be investigated. A caveat regarding interpretation of GLUT myocardial Western blots is that they will include some GLUT1 from red blood cells since red blood cells contain GLUT1 [31]. A preliminary report from Schwaiger et al. [32], using reverse transcriptase PCR in myocardial biopsies from patients with ischemic heart disease, described an increase in the ratio of GLUT1 and GLUT3 to GLUT4 mRNA, but no data are currently available regarding GLUT proteins.

The cell surface GLUT isoforms facilitate glucose entry into the cell. GLUT3 has been observed in the plasma membrane [19,33], and transports glucose into cells with high affinity [8,34]. GLUT3 protein was located intracellularly in myoblasts but also appeared on the plasma membrane in myotubes [19]. Translocation of GLUT3 (as well as GLUT1 and GLUT4) proteins from the light microsome fraction (i.e., intracellular) to the plasma membrane fraction occurred in L6 myotubes exposed to insulin, IGF-I and acylation-stimulating protein [18,20,22].

Glucose is critical for myocyte survival. Under normal conditions, myocytes can utilize multiple metabolic substrates. During ischemia and hypoxia, myocytes utilize relatively more glucose via anaerobic glycolysis because of the inability to metabolize fatty acids via β-oxidation [35,36]. Cellular availability of glucose is also important for maintenance of normal cellular integrity. Glycolysis not only preferentially inhibits ATP-sensitive potassium channels [37] but also is necessary to preserve myocardial calcium homeostasis during β-adrenergic stimulation [38]. Ischemic contracture begins when anaerobic glycolysis stops [39]. In addition, functional compartmentation of glycolytic versus oxidative metabolism has been demonstrated [40]. For these reasons, myocytes with impaired ability to take up glucose are at increased risk of cell death. Understanding regulation of myocardial GLUTs in order to maintain or enhance glucose uptake may help prevent cell death. Given the low K_m of GLUT3 protein, an increase in GLUT3 expression relative to expression of GLUT1 and GLUT4 may enable increased glucose entry into myocytes during conditions of increased demand for glucose. The regulation of human myocardial GLUT3 and its role in myocardial glucose transport remain to be elucidated.

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