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Molecular characterization and partial cDNA cloning of facilitative glucose transporters expressed in human articular chondrocytes; stimulation of 2-deoxyglucose uptake by IGF-I and elevated MMP-2 secretion by glucose deprivation

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Summary

Objective: Recent evidence suggests that human chondrocytes express several facilitative glucose transporter (GLUT) isoforms and also that 2-deoxyglucose transport is accelerated by cytokine stimulation. The aim of the present investigation was to determine if human articular chondrocytes express any of the recently identified members of the GLUT/SLC2A gene family and to examine the effects of endocrine factors, such as insulin and IGF-I on the capacity of human chondrocytes for transporting 2-deoxyglucose.

Design/methods: PCR, cloning and immunohistochemistry were employed to study the expression of GLUT/SLC2A transporters in normal human articular cartilage. The uptake of 2-deoxyglucose was examined in monolayer cultured immortalized human chondrocytes following stimulation with TNF-α, insulin and IGF-I. Levels of MMP-2 were assessed by gelatin zymography following glucose deprivation of alginate cultures.

Results: Using PCR we detected transcripts for eight glucose transporter isoforms (GLUTs 1, 3, 6, 8, 9, 10, 11 and 12) and for a fructose transporter (GLUT5) in human articular cartilage. Expression of GLUT1, GLUT3 and GLUT9 proteins in normal human articular cartilage was confirmed by immunohistochemistry. The uptake of 2-deoxyglucose was dependent on time and temperature, inhibited by cytochalasin B and phloretin, and significantly accelerated in chondrocyte cultures stimulated with IGF-I. However, 2-deoxyglucose uptake was unaffected by short and long-term insulin treatment, which ruled out a functional role for insulin-sensitive GLUT4-mediated glucose transport. Furthermore, secretion of MMP-2 was increased in alginate cultures deprived of glucose.

Conclusions: The data supports a critical role for glucose transport and metabolism in the synthesis and degradation of cartilage.

Key words: Chondrocyte, Cartilage, Glucose transport, GLUT, IGF-I, Insulin, MMP-2.

Introduction

Glucose is an essential metabolic substrate for articular cartilage and the intervertebral disk[1,2]. Chondrocytes are highly glycolytic resident cells of articular cartilage that metabolize glucose as a primary substrate for ATP production. Chondrocytes also incorporate glucosamine sulfate and other sulfated hexose sugars into newly synthesized extracellular matrix (ECM) glycosaminoglycans[3]. Chondrocytes therefore depend on glucose uptake and delivery to metabolic and biosynthetic pools. Members of the facilitative glucose transport/solute carrier 2A family of glucose/polyol transporters (GLUT/SLC2A) mediate the transport of glucose and related hexose sugars into mammalian cells[4] and, thus far, over a dozen GLUT family members have been identified in humans on the basis of sequence similarity[5] (see Table I for proposed physiological functions and tissue expression patterns of GLUT/SLC2A members). Independent studies from three laboratories suggest that chondrocytes express multiple isoforms of the GLUT/SLC2A family of glucose/polyol transporters[6–9]. In other tissues facilitative glucose transporter proteins are expressed in a tissue and cell-specific manner, exhibit distinct kinetic properties, and are developmentally regulated[10]. Recent studies show that in chondrocytes, facilitative glucose transporters are stimulated by proinflammatory cytokines such as interleukin 1 beta (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α)[7] which suggests that the increased glucose uptake observed in

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Table I
Summary of the extended GLUT family including gene names from recently revised GLUT nomenclature proposed by Joost and Thorens 2001 and Joost et al.*, 2002*

Protein	Gene name	cDNA	Expression	Proposed physiological function
GLUT1	SLC2A1	K03195	Erythrocytes, brain, cartilage	Housekeeping glucose transporter; ubiquitously expressed; basal glucose uptake for growth and development; transport across red cells, neuronal cells (blood brain barrier) and other tissues. GLUT1 expression appears to be up-regulated by glucose deprivation and prolonged hypoxia-ischemia. Expressed in almost all cultured cells
GLUT2	SLC2A2	J03810	Liver, pancreatic β cells, intestine	High-capacity, low affinity transporter expressed in the basolateral membrane of hepatocytes, pancreatic β cells and enterocytes
GLUT3	SLC2A3	J04069	Brain (neurons and glia), cartilage	Fast glucose transporter; Basal transport of glucose into many human cells; responsible for glucose uptake from cerebral fluid into parenchymal neurons; high affinity transporter specialized for glucose uptake where substrate concentrations are low
GLUT4	SLC2A4	M20747	Muscle, fat, heart, developing cartilage (chondroblasts, growth plate chondrocytes)	Insulin sensitive glucose transporter responsible for rapid glucose transport following insulin stimulation; implicated in diabetes
GLUT5	SLC2A5	J05461	Intestine, testis, kidney, cartilage	Fructose transporter expressed in the intestine; lower levels in other tissues. Responsible for transport of fructose and other hexoses
GLUT6 (alias GLUT9)	SLC2A6	Y17803	Spleen, leukocytes, brain, cartilage	Regulated by dynamin-dependent recycling between the plasma membrane and internal membranes
GLUT7	SLC2A7	CDNA not yet cloned	Unknown	Unknown
GLUT8 (alias GLUTX1)	SLC2A8	Y17801	Testis, blastocyst, brain, cartilage	Regulated by dynamin-dependent recycling between the plasma membrane and internal membranes; involved in pre-implantation development with other glucose transporters (GLUT1, GLUT3, GLUT5 and SGLT1). Down-regulated by glucose deprivation and prolonged hypoxia
GLUT9 (alias GLUTX)	SLC2A9	AF210317	Kidney, liver, cartilage	Glucose transporter, expressed in kidney, liver and cartilage
GLUT10	SLC2A10	AF321240	Heart, lung, liver, pancreas, skeletal muscle, placenta, kidney, cartilage	Low K_m (0.3 mM) glucose/galactose transporter; implicated in glucose metabolism and type 2 diabetes
GLUT11 (alias GLUT10)	SLC2A11	AJ271290	Heart, skeletal muscle, cartilage	Glucose transporter with several transcription variants (products of mRNA splicing)
GLUT12 (alias GLUT8)	SLC2A12	AY046419	Heart, skeletal muscle, prostate, cartilage	Expressed in skeletal muscle, adipose tissue, and small intestine; GLUT-12 may comprise a second insulin-sensitive glucose transport system.

Also included are cDNA accession numbers, proposed physiological function and limited tissue expression[3].

chondrocytes may be due to the mutual dependency of catabolic and anabolic pathways on regulated glucose transport. However, the contribution of anabolic endocrine factors such as insulin and insulin-like growth factor I (IGF-I) to the regulation of glucose transport in chondrocytes has yet to be studied. Accordingly, the objective of the present investigation was to determine if human articular chondrocytes express any of the recently identified members of the GLUT family and to identify the effects of insulin and IGF-I on the glucose uptake capacity of human chondrocytes.

Materials and methods

POLYMERASE CHAIN REACTION, CLONING AND SEQUENCING

Human articular cartilage cDNA libraries were obtained from Stratagene (La Jolla, CA, U.S.A.) and Clontech (Palo Alto, CA, U.S.A.). These cDNA libraries were prepared from pooled primary chondrocytes isolated from normal knee and hip joints of adult donors. Human-specific oligonucleotide primers were designed to amplify PCR products of discrete molecular weights corresponding to all known members of the GLUT/SLC2A family (Table II). PCR reactions were carried out essentially as described previously[8] using a PCR master mix (ABgene, U.K.) and consisted of an initial denaturation at 95°C for 1 min followed by 40 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min and a final extension step at 72°C for 1 min. PCR products were resolved in 1% agarose gels using a horizontal submarine gel electrophoresis system (Bio-Rad, Hemel Hempstead, U.K.) and DNA fragments were cloned using a pGEM®-T Easy cloning kit (Promega, Madison WI, U.S.A.) and sequenced commercially (MWG-Biotech AG, Ebersberg, Germany).

CELL CULTURE

Immortalized human chondrocytes (cell-line C20/A4; a gift from Dr M. B. Goldring, Beth Israel Deaconess Medical Center, Harvard University, Boston) were maintained in monolayer culture for glucose uptake studies. For Western blot analyses and MMP-2 assays, C20/A4 chondrocytes were maintained in alginate culture[11] at 37°C, 5% CO_2 in DMEM containing 1000 mg L^{-1} glucose supplemented with 10% fetal calf serum (Sigma, Poole, U.K.).

WESTERN BLOT ANALYSIS

Western blots of total protein derived from C20/A4 cell lysates were probed with polyclonal, human-specific antibodies to GLUT1, GLUT2, GLUT3 and GLUT4 (Chemicon International, Temecula, CA) and secondary alkaline phosphatase conjugated goat antirabbit IgG (Sigma). Premixed nitro blue tetrazolium (NBT) was used in conjunction with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as precipitating agent for alkaline phosphatase (Sigma).

IMMUNOHISTOCHEMISTRY

Formalin fixed, paraffin-embedded archival blocks of normal human articular cartilage graded according to the Mankin scale[12] were obtained with ethics committee approval from Manchester University. Sections were cut (6 μm thickness) onto microscope glass slides (BDH, U.K.) coated with 3-aminopropyl-triethoxysilane (APES; Sigma) or Vectabond reagent (Vector Laboratories, Peterbourough, U.K.). Polyclonal antibodies to GLUT1 were obtained from Dr S. Baldwin (University of Leeds, U.K.) for use in immunohistochemistry[13]. Cartilage sections were dewaxed in 100% xylene for 5 min and taken through a graded series (100%, 70% and 50%) of alcohol baths for 1 min each before antigen retrieval in the microwave oven in the presence of 10 mM citrate buffer (pH 6.0) for 12 min and 0.5% sodium dodecyl sulfate in phosphate buffer solution (PBS) for 10 min[14]. Any remaining endogenous alkaline phosphatase in the cartilage sections was blocked for one hour at room temperature (RT) by treatment with 1.25 mM levamisole solution (Vector Laboratories, Burlingame, CA, U.S.A.). Non-specific protein binding sites were blocked by addition of 10% normal goat serum in PBS (1 h RT). Sections were then incubated with polyclonal antibodies to GLUTs, 1, 3 and 9 (diluted 1:100 in PBS) for 24 h at 4°C and subsequently washed in PBS before a 2-h incubation at RT with goat antirabbit IgG conjugated to alkaline phosphatase. Sections were washed in PBS and alkaline phosphatase active sites were developed using Fast-Red TR/Naphthol AS-MX (Sigma) as precipitating agent for 25 min. Sections were counterstained with hematoxylin for 1 min and washed in distilled water before mounting in aqueous medium (H. D. Supplies, U.K.) for visualization under a light microscope. Photographs were taken using a Nikon Microphot-FX microscope fitted with a Nikon DXM1200 digital camera. Image analysis was carried out using Scion Image for Windows (version 4.0.2) based on NIH Image for Macintosh.

2-DEOXY-D-[2,6-^{3}H] GLUCOSE UPTAKE

Glucose transport was determined by measuring the uptake of non-metabolizable 2-deoxy-D-[2,6-^{3}H] glucose (Amersham/Pharmacia, Little Chalfont, U.K.) into monolayer cultured C20/A4 chondrocytes in the presence and absence of glucose transport inhibitors such as phloretin (0.2 μM ml^{-1}) and cytochalasin B (1 μM ml^{-1}). Additional 2-deoxy-D-[2,6-^{3}H] glucose uptake experiments were performed with cells pretreated/stimulated for 24 h with TNF-α (100 ng ml^{-1}), IGF-I (20 ng ml^{-1}), recombinant (long R^3) IGF-I (20 ng ml^{-1}) and insulin (12.5 μg ml^{-1}). Chondrocytes were cultured to 95% confluence in 24-well plates in DMEM containing 1000 mg ml^{-1} glucose and supplemented with 10% fetal calf serum. The wells were rinsed three times with PBS before the assay was performed for 35 min at room temperature in glucose, pyruvate and serum free DMEM (Sigma) containing 1 μCi ml^{-1} 2-deoxy-D-[2,6-^{3}H] glucose. The wells were then washed three times with ice-cold PBS before the cells were lysed in 0.5 ml of a cell lysis solution consisting of 0.5% sodium dodecyl sulfate and 0.5% Triton X-100 in PBS. 450 μl aliquots of cell lysates were transferred to 5 ml scintillation vials containing 3.55 ml of NACS104 scintillation cocktail for aqueous samples (Amersham/Pharmacia, Little Chalfont, U.K.). The uptake of the radiolabelled glucose was normalized to total cell protein content using a Bio-Rad detergent compatible (DC) protein assay. All uptake experiments were carried out in triplicate and repeated under identical conditions at least three times. The data is presented as percentage change in total 2-deoxy-D-[2,6-^{3}H] glucose uptake.

Table II

Oligonucleotide primer pairs used to detect the presence of mRNAs encoding members of the GLUT/SLC2A glucose transporter family in human articular cartilage by PCR (see Fig. 1)

Gene	Oligonucleotide primers 5′–3′ direction	PCR Product size (bp)	Homology to known sequences in GenBank
GLUT1 (SLC2A1)	(F) TCC ACG AGC ATC TTC GAG A (R) ATA CTG GAA GCA CAT GCC C	392	100% identical to nucleotides 1052 to 1450 of the *Homo sapiens* solute carrier family 2 (facilitated glucose transporter), member 1 (SLC2A1), mRNA (Accession number: XM_002033.4). Partial sequence submitted to GenBank as AY034633
GLUT2 (SLC2A2)	(F) CAC TGA TGC TGC ATG TGG C (R) ATG TGA ACA GGG TAA AGG CC	521	Not detected in human chondrocytes
GLUT3 (SLC2A3)	(F) TTC AAG AGC CCA TCT ATG CC (R) GGT CTC AGG GAC TTT GAA GA	457	100% identical to nucleotides 1180 to 1599 of the *Homo sapiens* solute carrier family 2 (facilitated glucose transporter), member 3 (SLC2A3), mRNA (Accession number: XM_049663.1). Partial sequence submitted to GenBank as AY034634
GLUT4 (SLC2A4)	(F) GGC ATG TGT GGC TGT GCC ATC (R) GGG TTT CAC CTC CTG CTC TAA	413	Not detected in human chondrocytes
GLUT5 (SLC2A5)	(F) CCC TTG ACG TTG CTG TGGT (R) ACA GTG ATG AAG AGC TGG GG	322	98% similarity to nucleotides 247 to 566 of the *Homo sapiens* solute carrier family 2 (facilitated glucose/fructose transporter), member 5 (SLC2A5), mRNA (Accession number: XM_045269.2). Partial sequence submitted to GenBank as AF479408
GLUT6 (SLC2A6)	(F) GGG TGT TCC TGG CCA CC (R) GCC TGG GGG AGC AAT CTC	350	PCR product not yet sequenced
GLUT7 (SLC2A7)		—	Not studied—cDNA not yet cloned
GLUT8 (SLC2A8)	(F) CAT GCT GCT TCT CAT GTG C (R) CAT GAG CCA GTT GGT GAG G	681	PCR product not yet sequenced
GLUT9 (SLC2A9)	(F) CATCAAGGCCTTTTACAATGAGT (R) AAGCCACCAATGAGGAGG	900	99% similarity to nucleotides 306 to 902 of the *Homo sapiens* solute carrier family 2 (facilitated glucose transporter), member 9 (SLC2A9), mRNA (Accession number: NM_020041.1). Partial sequence submitted to GenBank as AF421859
GLUT10 (SLC2A10)	(F) GGT CTT TGT CAG TGC CTT CT (R) GAG ATG TGC AAG TCA ATG GG	633	100% identical to nucleotides 1500 to 2131 of the *Homo sapiens* solute carrier family 2 (facilitated glucose transporter), member 10 (SLC2A10), mRNA (Accession number: XM_029983.1). Partial sequence submitted to GenBank as AF479407
GLUT11 (SLC2A11)	(F) GCG CTG CCG GCA TTG GTG GG (R) CAC CTA GGA GCT CCC TGA G	501	100% identical to nucleotides 151 to 649 of the *Homo sapiens* solute carrier family 2 (facilitated glucose transporter), member 11 (SLC2A11), mRNA (Accession number: XM_037696.1). Partial sequence submitted to GenBank as AF479409
GLUT12 Primer set A (SLC2A12)	(F) TCC CTC GTC ATT GGA GCC CTC CTT G (R) GCC ATC ACA GAG GAG CCA ATG CAG A	812	PCR product not yet sequenced
GLUT12 Primer set B (SLC2A12)	(F) TCC ATG GCT GGA AGT ACA T (R) TAA GTG TTC TGG CAC TAT C	1200	PCR product not yet sequenced
SGLT1 (SLC5A1)	(F) TCT TCG ATT ACA TCC AGT CCA (R) TCT CCT CTT CCT CAG TCA TC	521	Renal/intestinal sodium dependent glucose transporter. Not detected in human chondrocytes
β-actin (HUMACTA1)	(F) TTC AAC TCC ATC ATG AAG TGT GAC GTG (R) CTA AGT CAT AGT CCG CCT AGA AGC ATT	309	Housekeeping gene; used as internal PCR control

The primer list also includes the Na^+ dependent glucose carrier SGLT1 (SLC5A1) expressed in the intestine and kidney. Human β-actin (HUMACTA1) primers were included in all PCR experiments as an internal control. The homology of the sequenced PCR products to known sequences in the database was investigated using BLASTN 2.2.1 (http://www.ncbi.nlm.nih.gov/BLAST/). The partial GLUT cDNA sequences obtained from human chondrocytes were deposited in GenBank except for GLUT6, GLUT8 and GLUT12.

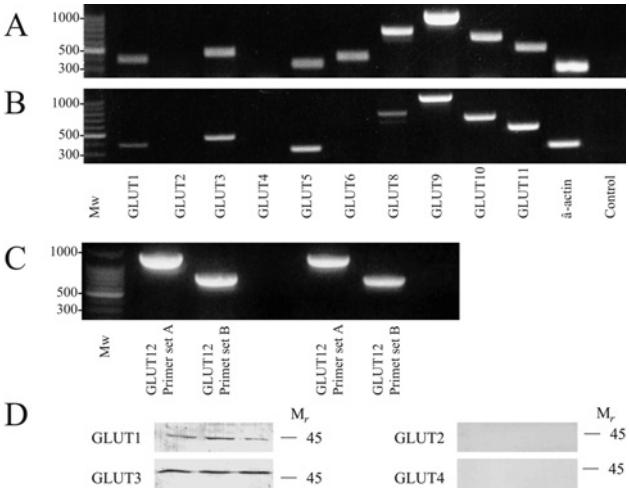

Fig. 1. PCR Evidence for up to nine facilitative glucose transporters in human articular cartilage. Panels A and B summarize the results of PCR experiments using two different human cartilage cDNA libraries confirm the expression of GLUT1, GLUT3, GLUT5, GLUT6, GLUT8, GLUT9, GLUT10, GLUT11 and GLUT12 in cartilage. The template was omitted in the control lanes shown in panels A and B. C. The presence of GLUT12 was confirmed using two different primer sets. Additional information about the sequences of the isoform specific PCR primers and the sizes of the PCR products may be found in Table I. D. Western blot showing expression of GLUT1 and GLUT3 and absence of GLUT2 and GLUT4 proteins in C20/A4 human chondrocyte-like cells maintained in alginate culture.

MMP-2 ZYMOGRAPHY

The effects of glucose transport inhibition (treatment with cytochalasin B) and glucose deprivation on the expression and secretion of active MMP-2 was determined by gelatin zymography. Alginate beads containing encapsulated C20/A4 chondrocytes were treated with cytochalasin B (10 μM) or deprived of glucose by incubation in glucose-free DMEM for 24, 36 and 72 h in 24 well plates containing 2 ml of serum-free DMEM culture medium. At the end of each 24-h period, aliquots of the culture medium supernatant were subjected to SDS-PAGE and zymography as previously described[15]. The zymogram gels were scanned using a dedicated flatbed scanner and the densities of active MMP-2 bands quantified using Scion Image for Windows (version 4.0.2).

STATISTICAL ANALYSIS

Comparisons between control, insulin, IGF-I, IGF-I R^3 and TNF-α stimulated glucose uptake were made by means of the Student's *t*-test.

Results

HUMAN ARTICULAR CHONDROCYTES EXPRESS UP TO NINE GLUT/SLC2A ISOFORMS

The results of the PCR experiments on two different human cartilage cDNA libraries confirmed that GLUT1 (SLC2A1), GLUT3 (SLC2A3), GLUT5 (SLC2A5), GLUT6 (SLC2A6), GLUT8 (SLC2A8), GLUT9 (SLC2A9), GLUT10 (SLC2A10), GLUT11 (SLC2A11) and GLUT12 (SLC2A12) are expressed in human cartilage (Fig. 1). In both cDNA libraries examined, the transcripts corresponding to the sodium-dependent glucose transporter SGLT1 and the facilitative glucose carriers GLUT2 and GLUT4 were undetectable as we and others have previously shown[3,7–9]. Human prostate and intestine cDNA libraries were used as positive controls to ensure that the primers designed against SGLT1, GLUT2, and GLUT4 produced the expected PCR products (results not shown). The only difference observed between the two cDNA libraries used was expression of GLUT6. We found GLUT6 in only one cDNA and further studies may be necessary to confirm this finding. Expression of GLUT7 was not investigated as the cDNA for the SLC2A7 gene has not yet been cloned. The PCR experiments were repeated nine times with each GLUT primer set. Purification, cloning and sequencing of the PCR products shown in Fig. 1(A) confirmed expression of GLUT1, GLUT3, GLUT5, GLUT9, GLUT10 and GLUT11 in human chondrocytes. Partial sequences obtained were deposited in GenBank using the web-based program BankIt (see Table I). We performed PCR detection of GLUT12 using two sets of primers; primer set A produced a 812 base pair product and primer set B produced a 1.2 kilobase product characteristic of GLUT12 [see Fig. 1(C)]. The second primer set has also been used to detect GLUT12 transcripts in human breast cancer cell line where GLUT12 is abundantly expressed (S. Rogers, personal communication).

WESTERN BLOT EVIDENCE FOR GLUT1 AND GLUT3 EXPRESSION IN C20/A4 CHONDROCYTES

Western blot analysis demonstrated that C20/A4 chondrocytes expressed GLUT1 and GLUT3. We were unable to detect GLUT2 and GLUT4 proteins, further confirming the results obtained by PCR [Fig. 1(D)].

IMMUNOHISTOCHEMICAL EVIDENCE FOR GLUT1, GLUT3 AND GLUT9 IN NORMAL HUMAN CARTILAGE

To further substantiate the results obtained by PCR, we employed immunohistochemistry to demonstrate that GLUT1, GLUT3 and GLUT9 proteins are present in normal human articular cartilage. For this purpose we used well-characterized isoform specific antibodies to GLUT1, GLUT3 and GLUT9 to show that these proteins were detectable in human chondrocytes *in situ* (Fig. 2).

TIME AND TEMPERATURE DEPENDENCE OF 2-DEOXYGLUCOSE UPTAKE

Uptake studies in immortalized human chondrocytes indicated that these cells express functional glucose transporters and that the uptake of 2-deoxy-D-[2,6-^{3}H] glucose into C20/A4 chondrocytes was time and temperature dependent (Fig. 3). The rate of 2-deoxy-D-[2,6-^{3}H] glucose uptake was higher at 37°C than at 20°C. These results were consistent with a facilitative, GLUT-mediated process for glucose transport[16].

INHIBITION OF GLUCOSE UPTAKE BY CYTOCHALASIN B AND PHLORETIN

Baseline 2-deoxy-D-[2,6-^{3}H] uptake in control chondrocytes was calculated as 100%. The uptake of 2-deoxy-D-

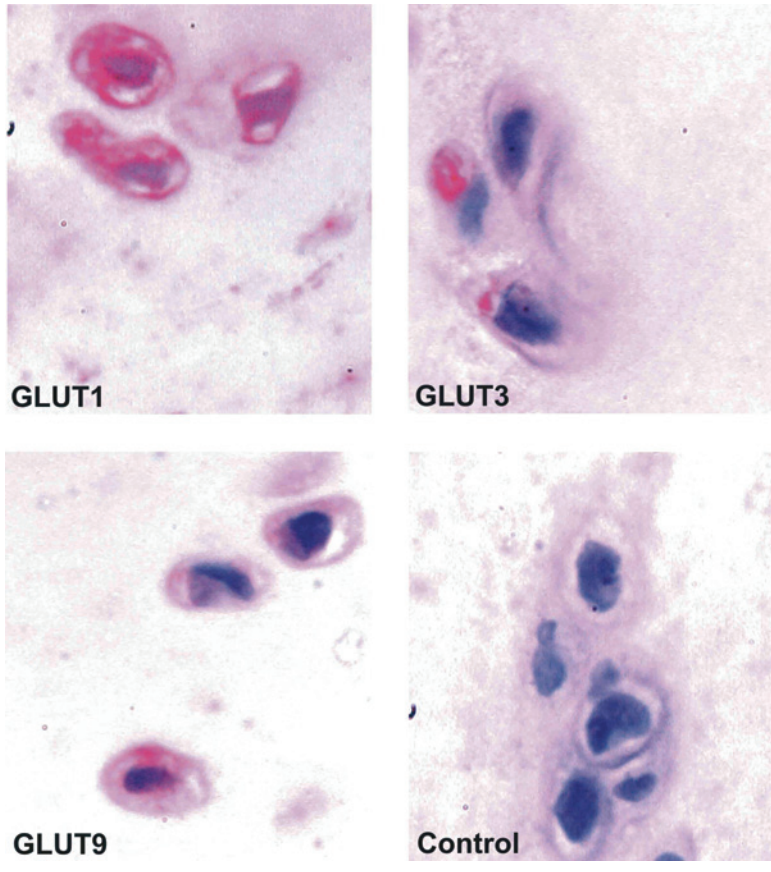

Fig. 2. Immunohistochemical evidence for expression of GLUT1, GLUT3 and GLUT9 glucose transporters in normal human articular cartilage (original magnification ×400). The immunostaining for the GLUT proteins is indicated by the red substrate and the cell nuclei were counterstained with hematoxylin. The primary antibody was omitted in the control panel, which shows a section of human cartilage exposed only to secondary alkaline phosphatase conjugated antibody.

[2,6-^{3}H] glucose was significantly reduced (P<0.005) by up to 90% in the presence of the glucose transport inhibitors cytochalasin B and phloretin [Fig. 4(A)]. When used in combination, phloretin and cytochalasin B reduced 2-deoxy-D-[2,6-^{3}H] glucose uptake by more than 95% (P<0.005). In all cases, a statistically significant difference was observed between the control group and groups incubated with cytochalasin B and phloretin. These compounds are potent inhibitors of facilitative glucose transport providing additional evidence that glucose uptake in chondrocytes is GLUT-mediated.

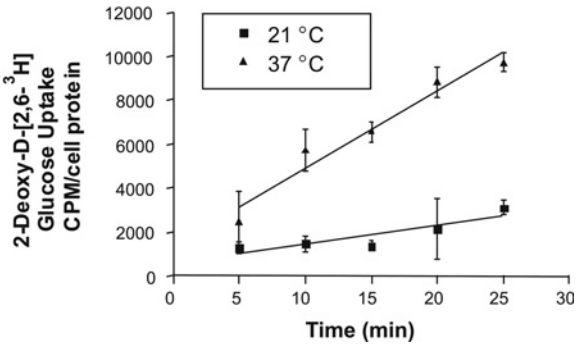

Fig. 3. Time and temperature dependence of 2-deoxy-D-[2,6-^{3}H] glucose uptake by C20/A4 chondrocytes. Error bars indicate standard deviation of the means (n=3).

GLUCOSE UPTAKE IS STIMULATED BY IGF-I AND TNF-α BUT NOT BY INSULIN

To test the effects of insulin, IGF-I and TNF-α on 2-deoxy-D-[2,6-^{3}H] glucose uptake by C20/A4 chondrocytes, cells maintained in 24-well plates were stimulated for 24 h with the above before glucose transport was assayed by 2-deoxy-D-[2,6-^{3}H] uptake. Again, baseline 2-deoxy-D-[2,6-^{3}H] uptake in unstimulated chondrocytes was considered as 100%. The data presented in Fig. 4(B) indicates that insulin treatment resulted in a small but statistically insignificant increase in glucose uptake. In contrast IGF-I and TNF-α both resulted in a statistically significant (P<0.05) increase of up to 68% and 72% in glucose uptake respectively. Short-term administration of insulin (30 min) did not increase glucose uptake (data not shown).

GLUCOSE DEPRIVATION RESULTS IN ELEVATED MMP-2 SECRETION

The alginate culture system was employed to determine the effects of inhibition of glucose transport and removal of glucose from the culture medium on chondrocyte behavior.

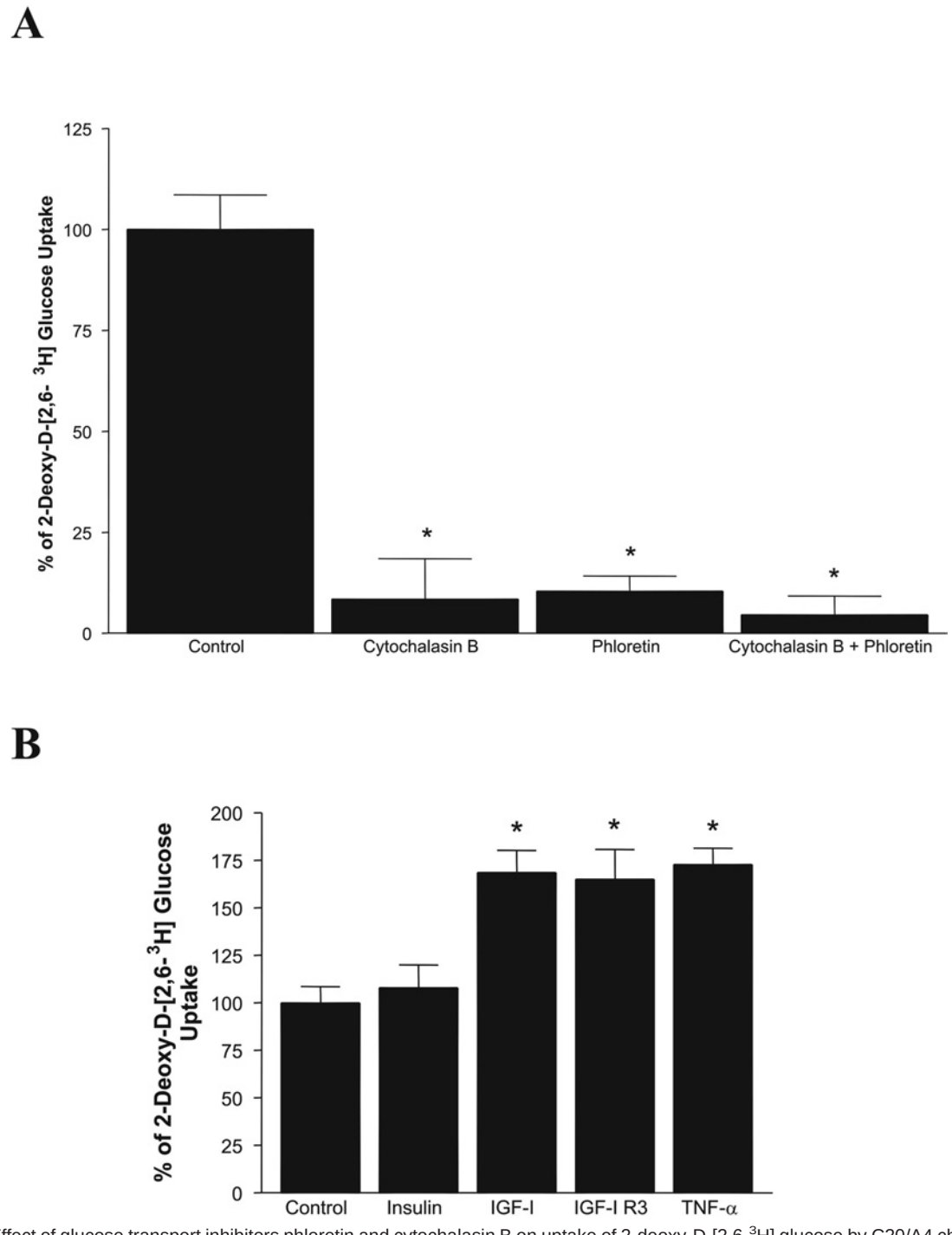

Fig. 4. A. Effect of glucose transport inhibitors phloretin and cytochalasin B on uptake of 2-deoxy-D-[2,6-^{3}H] glucose by C20/A4 chondrocytes grown in 24 well plates. 2-deoxy-D-[2,6-^{3}H] glucose uptake in control (baseline) chondrocytes was compared with cells incubated with 0.2 μM ml^{-1} phloretin and 1 μM ml^{-1} cytochalasin B singly or combination throughout the assay period. B. Effects of insulin, insulin-like growth factor (IGF-I) and TNF-α on 2-deoxy-D-[2,6-^{3}H] glucose uptake by C20/A4 chondrocytes. Chondrocytes were stimulated with insulin (12.5 μg ml^{-1}), TNF-α (20 ng ml^{-1}) and recombinant (long R^3) IGF-I (20 ng ml^{-1}) for a period 24 h at 37°C before facilitated glucose transport was measured by 2-deoxy-D-[2,6-^{3}H] uptake. Baseline 2-deoxy-D-[2,6-^{3}H] uptake in un-stimulated chondrocytes (control) was considered as 100%. Error bars indicate standard errors of the means (n=3). In cases where a statistically significant difference between the experimental group and a control group was found the bar is labeled with *.

We used gelatin zymography to assay the levels of secreted, active MMP-2 in supernatants of alginate-encapsulated chondrocytes treated with cytochalasin B or cells deprived of glucose for up to 72 h. Control alginate cultures showed a moderate rise in secreted MMP-2 levels over a 3-day period. However, secretion of active MMP-2 was significantly increased (P<0.05) in supernatants of alginate cultures deprived of glucose (Fig. 5).

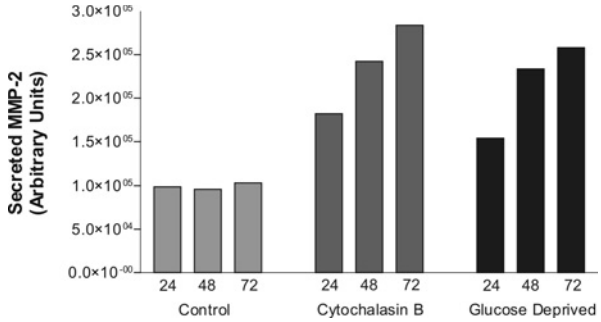

Fig. 5. Effects of glucose transport inhibition (treatment with cytochalasin B) and glucose deprivation on the expression and secretion of active MMP-2 in C20/A4 chondrocytes in alginate culture. Inhibition of glucose transport and glucose deprivation for up to 72 h significantly increased MMP-2 secretion by chondrocytes compared to the control group.

Discussion

Degenerative disorders of load-bearing articular cartilage and intervertebral disc are generally characterized by disequilibria between ECM repair and degradative processes, with the former not keeping pace. This results in a loss of proteoglycans, type II collagen, and other ECM components as a result of elevated matrix metalloprotease and aggrecanase activity[17]. Recent studies suggest that cartilage degeneration occurs concomitantly with endocrine dysfunction, glucose imbalance and diabetes mellitus[18–20]. In healthy cartilage, IGF-I promotes differentiated cellular functions, which include stimulation of ECM repair. The reduced growth and repair observed in osteoarthritis (OA) may be related to an inability of IGF-I to exert its anabolic effect on chondrocytes. There is also an age-related decline in the chondrocyte response to IGF-I and chondrocytes from human OA joints do not respond well to IGF-I stimulation[21]. This anomaly known as the 'IGF-I-resistant state' implicates insulin/IGF-I signalling and glucose metabolism in the pathogenesis of OA[22].

Glucose is an important metabolite and structural precursor for articular cartilage. The steady supply and transport of physiological levels of glucose has significant consequences for chondrocyte viability, ECM synthesis and the development and functional integrity of cartilage[23]. Articular cartilage is a poorly vascularized and highly glycolytic tissue, which produces large quantities of lactic acid. This situation is further exacerbated by low oxygen tensions and ongoing anaerobic glycolysis by chondrocytes. Therefore, even modest changes in glucose concentrations in the ECM microenvironment could impair IGF-I-mediated anabolic activities and promote a variety of joint pathologies[18,24]. Therefore, healthy cartilage depends on a functional endocrine system that finely regulates blood glucose and, hence, synovial glucose levels.

We recently presented a novel hypothesis implicating glucose transporters and enzymes responsible for glycolysis and energy metabolism in cartilage physiology and pathophysiology[3]. Facilitative glucose transporters represent the rate-limiting step for glucose uptake across the chondrocyte membrane. GLUTs also provide the means for accelerated glucose transport and enhanced substrate utilization in response to stimulation by pro-inflammatory, catabolic cytokines, including TNF-α, IL-6 and IL-1β[25,26] and to chemokines such as connective tissue activating peptide III[27].

In this study we have shown, for the first time, that glucose transporters exist in multiple molecular 'isoforms' and that the glucose transporting capacity of chondrocytes is increased by IGF-I stimulation. Thus, it would appear that stimulated glucose transport and its subsequent utilization represent important components of the chondrocyte response to both anabolic and catabolic mediators[25,27]. Stimulated glucose transport and upregulation of GLUT proteins may be an early and sustained event in inflammatory processes in cartilage. Further studies are needed to determine which GLUT isoforms are involved in the IGF-I response.

Our PCR studies partially confirm data from studies on rat growth plate chondrocytes where GLUTs 1, 2, 3, 4 and 5 are expressed[6], with the exception that we did not detect GLUT2 and GLUT4. These results and the absence of GLUT2 and GLUT4 are also consistent with recent observations in human articular chondrocytes[7]. However, in contrast to this earlier report, our data suggests that GLUT1, GLUT3 and GLUT9 isoforms are constitutively expressed in human chondrocytes. Facilitative glucose transporters exhibit variable hexose affinity and tissue-specific expression and these characteristics contribute to specialized metabolic properties of various cell types within the body. This multitude of GLUT isoform diversity in chondrocytes suggests that the transmembrane uptake of glucose, fructose and other related hexose sugars is highly specialized and is achieved by the concerted action of multiple members of the GLUT/SLC2A gene family. We suggest that this GLUT isozyme diversity in chondrocytes may reflect a cartilage-specific requirement for 'fast' (i.e. GLUT3 and GLUT10) and baseline (GLUT1) glucose transporters that operate more efficiently at low substrate concentrations under physiological conditions[3]. We found no evidence for the classical, acutely insulin-responsive GLUT4. However, we were able to demonstrate the presence of the GLUT8 and GLUT12 glucose transporters, which have been proposed to be insulin-responsive in the long-term but not acutely insulin-sensitive[10,28]. The absence of the GLUT4 glucose transporter in mature cartilage rules out a role for acute regulation of glucose transport by insulin in mature, fully developed human articular cartilage. Nonetheless, insulin and IGF-I regulate glucose uptake in chondrocytes over a longer time period.

GLUT9 is most abundantly expressed in the liver and the kidney[29]—both gluconeogenic tissues. Since chondrocytes also possess the capacity for gluconeogenesis via the glyoxylate pathway[30], it is possible that GLUT9 may be involved in the transport of glucose across the endoplasmic reticulum and Golgi apparatus. The precise physiological role of GLUT9 in cartilage is unknown yet it may be implicated in intracellular glucose transport, glycosylation or glycogenesis. GLUT12 has been detected in embryonic rat cartilage[36] and this study suggests that its expression appears to be maintained in adult articular cartilage.

Chondroblasts and mature chondrocytes are derived from multi-potential mesenchyme stem cells, which include bone marrow stromal cells of mesodermal origin. These cells can also differentiate into other cell types including myocytes, adipocytes, fibroblasts and osteoblasts[31–33]. In this context, these cell types share a number of phenotypic characteristics as a result of their shared lineage[34]. Although adult articular chondrocytes express the leptin receptor (a product of the obese *Ob-R* gene and a marker of end-stage differentiation of stromal cells to adipocytes)[35] they do not express the classic adipocyte marker

GLUT4[6,8,9]. Further basic research is required to determine if cells in the mesenchymal lineage share expression of other members of the GLUT/SLC2A family.

Finally, we have demonstrated that glucose deprivation increases MMP-2 secretion. Similar results were obtained by inhibition of glucose transporters or removal of glucose from the culture medium. Therefore cell stress caused by reduced glucose concentrations in the ECM not only impair IGF-I-mediated anabolic activities but could also promote further ECM degradation by secretion of active matrix proteases.

A clearer knowledge of chondrocyte nutrition and the regulation of transport systems responsible for nutrient uptake in chondrocytes in health and disease may reveal underlying metabolic disturbances that are directly responsible for cartilage degradation in OA and other arthropathies[19,24]. This understanding may lead to novel therapies to prevent and treat degenerative joint disease as well as contribute to the development of disease-modifying drugs. Clearly, this is an emerging field and future studies are required to compare the expression pattern of glucose transporters in normal and OA cartilage. It remains to be determined whether glucose transport in chondrocytes is influenced by transforming growth factor beta (TGF-α), connective tissue growth factor (CTGF) and circulating hormones (growth hormone, leptin) as glucose transport appears to be regulated by IGF-I and the proinflammatory cytokines TNF-α, IL-6 and IL-1β[7,25,26].

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