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Glucosamine modulates chondrocyte proliferation, matrix synthesis, and gene expression

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Summary

Objective: To investigate the effects of glucosamine (GlcN) on chondrocyte proliferation, matrix production, and gene expression for providing insights into the biochemical basis of its reported beneficial effects in osteoarthritis (OA).

Methods: Dose-dependent effect of GlcN on cell morphology, proliferation, cartilage matrix production and gene expression was examined by incubating primary bovine chondrocytes with various amounts of GlcN in monolayers (2D) and in cell-laden hydrogels (3D constructs). Histology, immunofluorescent staining and biochemical analyses were used to determine the effect of GlcN on cartilage matrix production in 3D constructs. The impact of GlcN on gene expression was evaluated with real-time polymerase chain reaction (PCR).

Results: GlcN concentration and culture conditions significantly affected the cell behavior. Quantitative detection of matrix production in cell-laden hydrogels indicated a relatively narrow window of GlcN concentration that promotes matrix production (while limiting cellular proliferation, but not cell viability). Notably, GlcN enhanced cartilage specific matrix components, aggrecan and collagen type II, in a dose-dependent manner up to 2 mM but the effect was lost by 15 mM. Additionally, GlcN treatment up-regulated transforming growth factor-β1 (TGF-β1) mRNA levels.

Conclusion: Results indicate that culture conditions play a significant role in determining the effect of GlcN on chondrocytes, explaining both the previously reported beneficial and deleterious effects of this sugar. The ability of GlcN to alter TGF-β1 signaling provides a biochemical mechanism for GlcN activity on chondrocytes that up to now has remained elusive. The observed anabolic effect of optimal GlcN concentrations on chondrocytes may be useful in formulating effective cartilage repair strategies.

Key words: Cartilage repair, Glucosamine, Osteoarthritis.

The degenerative disease osteoarthritis (OA) is a manifestation of an imbalanced synthesis of articular cartilage (AC) matrix and the associated growth factors[1,2]. Currently available therapies for improving joint health and OA include the use of anti-inflammatory and pain relieving drugs for symptomatic relief. The use of potentially chondro-protective agents such as glucosamine (GlcN) and chondroitin sulfate (CS) has also been explored to treat OA[3,4]. These compounds are sold in the US as food supplements, while they are prescribed as drugs in Europe. CS is a major component of glycosaminoglycan (GAG) while GlcN is a constituent of the disaccharide building blocks of CS. It has been hypothesized that the dietary supplementation of these components stimulates cartilage regeneration. However, various studies that have investigated the effect of these components on cartilage regeneration report mixed results; some promising, and others not, making this subject highly controversial. Nonetheless, most of the *in vivo* clinical studies have demonstrated the efficacy of GlcN in alleviating symptoms of OA[5–7]. For instance, McAlindon *et al.*, have carried out a meta-analysis combined with systematic quality assessment of clinical trials treating knee and hip OA using GlcN and CS to evaluate their beneficiary effect on OA[3]. Their analyses indicate a moderate to large beneficial effect of these nutrients on OA. Other double-blind and placebo-controlled randomized clinical trials also show that GlcN is capable of relieving osteoarthritic pain[5,7–10].

Although the oral administration of GlcN is considered to be promising in the treatment of OA, its structure-modifying effect on human AC is not understood at the cellular (chondrocyte) and tissue (cartilage explants) levels[11]. Furthermore, *in vitro* studies of chondrocytes or cartilage explants treated with GlcN are conflicting; some studies indicating the inhibition of cartilage destruction in the presence of GlcN[12], while other studies finding an adverse effect of GlcN on the biomechanical properties of cartilage explants or cell viability[13]. Investigations on the serum and synovial pharmacokinetics of dietary GlcN demonstrated that only low levels of GlcN reach into the joint[14–16]. For instance, Laverty *et al.* have shown that the concentration of GlcN in serum and synovial fluid reached only 6 μM and 0.3–0.7 μM, respectively, after nasogastric intake of 20 mg/kg/day of GlcN in equine model[14].

Oral as well as intravenous administration of GlcN has also been shown to be effective in various animal models[17,18]. Noyszewski *et al.* have reported that exogenously supplied GlcN facilitates the production of CS

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moieties of GAG in AC explants[19]. Fenton *et al.*, demonstrated that GlcN can prevent the experimentally induced equine cartilage degradation[20]. These studies indicate that exogenous GlcN may be a good precursor for GAG synthesis, whereas, other studies suggest that GlcN prevents the degradation of proteoglycans by inhibiting the nitric oxide synthesis in the presence of externally introduced inflammatory cytokines such as interleukin-1β (IL-1β)[21–24].

In this study, we aim to identify the amount of GlcN that has significant effect on chondrocytes' biosynthetic activity and hence the matrix production. We provide a systematic evaluation of the effects of GlcN on primary bovine articular chondrocytes (BAC), both in monolayer (2D) and three-dimensional (3D) culture conditions and demonstrate that the response of the cells is dramatically different in the two culture conditions. Our findings emphasize the need for a realistic cell culture model system and the use of an optimal GlcN concentration to obtain physiologically-relevant results. Additionally, the GlcN mediated up-regulation of transforming growth factor-β1 (TGF-β1) provides an explanation for previously-failed attempts to account for the effects of this sugar through metabolic flux considerations[25].

Materials and methods

MATERIALS

GlcN–HCl was obtained form Sigma–Aldrich (St. Louis, MO). Culture medium (chondrocyte medium) was prepared by dissolving the desired amount of GlcN in chondrocyte medium so as to attain the required final concentrations of GlcN in millimoles (mM).

ISOLATION OF BACs

Cartilage slices were surgically dissected from the patellofemoral groove and femoral condyles of bovine legs from 5–8 week old calves (Research 87, Marlboro, MA) as described earlier[26]. Small pieces of cartilage tissues were incubated at 37 °C and 5% CO_2 in Dulbecco's modified eagle medium (DMEM, Gibco, Grand Island, NY, USA) containing 0.2% collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA) and 5% fetal bovine serum (Gibco) for 16 h using an orbital shaker at 120 rpm. The resulting cell suspensions were then filtered through 70 μm nylon filters (Cell Strainer; Falcon, Franklin Lakes, NJ, USA) and washed with phosphate buffered saline (PBS) containing 1% penicillin streptomycin. The cell number was measured using a Z2 coulter counter and Size analyzer (Beckman Coulter Inc., Palo Alto, CA, USA).

PHOTOENCAPSULATION OF BACs IN HYDROGELS (3D CULTURE)

Poly(ethyleneglycol)-diacrylate (PEGDA; procured from Nectar, Huntsville, AL) was dissolved in sterile PBS, containing 1% penicillin streptomycin, to achieve 10% (w/v) precursor solution. The photoinitiator Irgacure D2959 (Ciba Specialty Chemicals, Tarytown, NY) was added to the PEGDA solution and mixed thoroughly to achieve a final 0.05% w/v of photoinitiator concentration. Chondrocytes were suspended within the precursor solution (20 million cells/ml) and then transferred into a sterile cylindrical mold followed by exposing them to long wavelength 365 nm light at 4.5 mW/cm^2 (Glowmark Systems, Upper Saddle River, NJ), for 5 min to achieve complete gelation. The cell-laden hydrogels (3D constructs) were then transferred into dishes and cultured in the presence of varying GlcN concentrations. The 3D constructs were harvested at different time intervals and analyzed to evaluate cell proliferation, matrix production, gene expression and phenotypic stability.

GROWTH KINETICS

Primary chondrocytes were plated in cell culture dishes at an initial cell density of 5000 cells/cm^2 and simultaneously exposed to chondrocyte medium [DMEM (Gibco, Invitrogen), supplemented with 10 mM HEPES (Gibco, Invitrogen), 0.4 mM L-proline (Sigma, St. Louis, MO), 50 μg/ml ascorbic acid (Sigma), 10% fetal bovine serum (FBS, Qualified), 0.1 mM non-essential amino acid (Gibco, Invitrogen), and 1% penicillin streptomycin] containing 1, 2, 5, 10 and 15 mM GlcN, while control cultures were not exposed to GlcN. To evaluate the effect of GlcN on cell morphology, the samples were observed periodically through an optical microscope after seeding of the cells and their subsequent exposure to GlcN. Cells from six dishes were trypsinized at a specific time each day and counted using a Z2 Coulter Particle Count and Size analyzer. The initial population doubling time (P_2) was estimated from the proliferation data in the first 3 days after plating the cells. Assuming an exponential growth of the cells, the population data were fitted to the following equation

$$P = P_0 \exp(\lambda t) \quad (1)$$

where P_0 is the number of plated cells at time $t = 0$, and λ is an adjustable parameter. The population doubling time P_2 is then given by

$$P_2 = \ln(2)/\lambda. \quad (2)$$

Proliferation rate of chondrocytes in the presence of varying GlcN concentration was also analyzed through a water soluble tetrazolium salt (WST)-1 assay. The cells were plated in a 96-well plate and cultured in the presence of various amounts of GlcN. Ten micro liters of WST-1 cell proliferation reagent (Roche Molecular Biochemicals, Hannheim, Germany) were added into the cell suspension and incubated at 37 °C for 3 h. The WST-1 derived precipitate, produced by metabolically active cells in the culture, was quantified by a multi-well plate reader (μQuant, BIO-TEK Instruments, Winooski, VT) at A_{450} as per the manufacturer's protocol. The viable cells for each condition were determined by WST-1 absorbance. To examine cell proliferation, the average absorbance of wells containing the specific medium and WST-1 (considered as the background) was subtracted from the absorbance of the wells containing the cells, the medium, and WST-1.

To further understand the cellular response to varying GlcN concentrations in culture medium, three different experiments were designed: (1) cells were mixed with chondrocyte medium already containing GlcN and plated, (2) cells were cultured in chondrocyte medium for 3 days before their exposure to GlcN, and (3) cells were cultured for 6 days in chondrocyte medium and then exposed to GlcN. Henceforth, we refer to these three culture conditions as D0, D3, and D6, respectively. Control cultures were maintained without GlcN exposure. All groups were cultured for a total of 11 days and the medium was changed twice per week. The trypsinized cells were counted using a Z2 coulter counter and cell viability was determined as mentioned below.

CELL VIABILITY ASSAY

A hemocytometer-based trypan blue dye exclusion method was used to determine cell viability of monolayer cultured chondrocytes[27]. Briefly, 20 μl of cell suspension was mixed thoroughly with 20 μl of trypan blue (0.4% trypan blue in 0.85% saline). A small amount of this suspension was loaded onto the hemocytometer and the living (unstained) and dead (stained) cells were counted using a microscope. Cell viability was also analyzed using Calcein AM/EthD-I Live/Dead Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR). Calcein AM specifically stains living cells via their intracellular esterase activity while EthD-I stains dead cells that have lost plasma membrane integrity. The reagents were diluted according to the manufacturer's protocol and incubated for 30 min in serum-free medium before exposure to fluorescent light.

To examine the cell viability of 3D constructs after photopolymerization, samples were cut into thin slices and stained using Calcein AM/EthD-I Live/Dead Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR) as mentioned above. Cell viability of 3D constructs cultured with varying GlcN concentration was evaluated by tunnel staining. Paraffin-embedded 6 week cultured 3D construct sections were stained for apoptotic cells by TdT-mediated dUTP nick-end labeling (tunnel) assay according to the supplier's instruction. The percentage of viable cells was determined by counting the stained cells relative to the total cells (stained with DAPI) presented in six different fields.

HISTOLOGY AND IMMUNOFLUORESCENT STAINING

Hydrogel constructs were fixed overnight in 4% paraformaldehyde in PBS (pH 7.4) at 4°C and transferred to 70% ethanol until processing. Constructs were embedded in paraffin, and cut into 5 μm sections that were stained with hematoxylin and eosin, and Safranin-O/fast green. Immunofluorescent staining was performed according to the manufacturer's protocol (Zymed Laboratories, San Francisco, CA). Polycolonal rabbit antibodies against mouse types I and II (RDI, Flanders, NJ) were used with 1:40 to 1:100 dilutions.

BIOCHEMICAL ASSAY

The lyophilized 3D constructs were crushed using pellet pestle mixer (Kimble/Kontes) and digested in papainase solution (construct/1 ml papainase solution; 125 μg/mL; Worthington Biomedical, Lakewood, NJ) for 18 h at 60°C. The DNA content was determined using Hoechst 33258 dye[28]. The GAG content characterized by CS was measured using dimethylene blue (DMMB) spectrophotometric assay at A_{525}[29]. CS in de-ionized water was used as standard. Total collagen content was determined by measuring the hydroxyproline content of the constructs after acid hydrolysis and reaction with p-dimethylaminobenzaldehyde and chloramine-T[30].

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR) AND REAL-TIME PCR

Total RNAs were extracted from 2D monolayer cultures and 3D constructs using TRIzol (Invitrogen, Carlsbad, CA) following the manufacturer's instruction. Two micrograms of total RNA per 20 μl of reaction volume were reverse transcribed into cDNA using the SuperScript First-Strand Synthesis System (Invitrogen). Real-time PCR reactions were performed and monitored using the SYBR Green PCR Mastermix and the ABI Prism 7700 Sequence Detection System (Perkin Elmer/Applied Biosystems, Rotkreuz, Switzerland). cDNA samples (2 μl for total volume of 25 μl per reaction) were analyzed for genes of interest and for reference β-actin. The level of expression of each target gene is then calculated as $2^{-\Delta\Delta C_T}$, as previously described[31]. Each sample was repeated three times for each gene of interest. RT-PCR was performed at 95°C for 2 min followed by 34 cycles of 30 s denaturation at 95°C, 30 s annealing at the primer specific temperature, and 1 min elongation at 72°C. PCR products were verified by electrophoresis. The PCR primers are listed in Supplementary Table 1.

STATISTICAL ANALYSIS

All results are presented as averages and standard deviations ($n = 6$). Statistical significance was determined by one way single factor analysis of variance (ANOVA; in the Microsoft Excel package) and *post hoc* Tukey tests and set as $P < 0.05$.

Results

EFFECT OF GlcN ON CELL MORPHOLOGY AND PROLIFERATION IN 2D MONOLAYER CULTURES

To understand the dose-dependent effect of GlcN on cell morphology and growth kinetics, primary chondrocytes were cultured in the presence of varying GlcN concentrations. The cell morphology changed with GlcN exposure when cells were plated and exposed to GlcN at the same time, i.e., D0 culture condition [Fig. 1(A)]. Without GlcN, the cell number increased after plating and all cells had a fibroblastic morphology. However, upon incubation with GlcN, the cell number decreased and a larger fraction of cells retained the original "spherical" morphology, as shown in [Fig. 1(A)]. Cells that were exposed to GlcN proliferated slowly, requiring a longer time to achieve 100% confluency as compared to their GlcN-deficient counterparts. GlcN-induced cell death was observed at higher GlcN concentrations; at 10 and 15 mM GlcN, cell death occurred after 4 and 3 days, respectively, as determined by trypan blue staining and live–dead analysis. However, if cells were allowed to adhere onto the culture dish before their exposure to GlcN (D6 culture conditions), significantly higher concentrations of GlcN (10 and 15 mM) could be tolerated [Figs. 1(B) and 2]. Cells that were cultured for 6 days prior to GlcN exposure achieved confluency in 11 days, with a cell number comparable to cells cultured in the absence of GlcN [Figs. 1(B) and 2]. Nonetheless, a decrease in cell proliferation with increasing GlcN concentration was observed in all systems (Fig. 2).

The early phase growth kinetics of chondrocytes exposed to various amounts of GlcN is shown in [Fig. 3(A)]. The effect of GlcN on cell proliferation was more dramatic at day 3 as compared to 1 and 2 days after plating. The calculated population doubling times (P_2) based on these initial proliferations [using Eqs. (1) and (2)] show a subtle increase in P_2 with GlcN at low concentrations [Fig. 3(B)]. Beyond 2 mM, P_2 shows a marked increase with GlcN concentration. A similar trend was observed with the WST-1 cell proliferation assay vs GlcN concentration. In WST-1 assay, the absorbance of the WST-1 precipitate decreased with increasing GlcN concentration, indicating a decrease in cell number. Absorbance values at days 3 and 4 indicate cell death when WST-1 solutions were added to culture medium containing 15 and 10 mM GlcN, respectively [Fig. 3(C)].

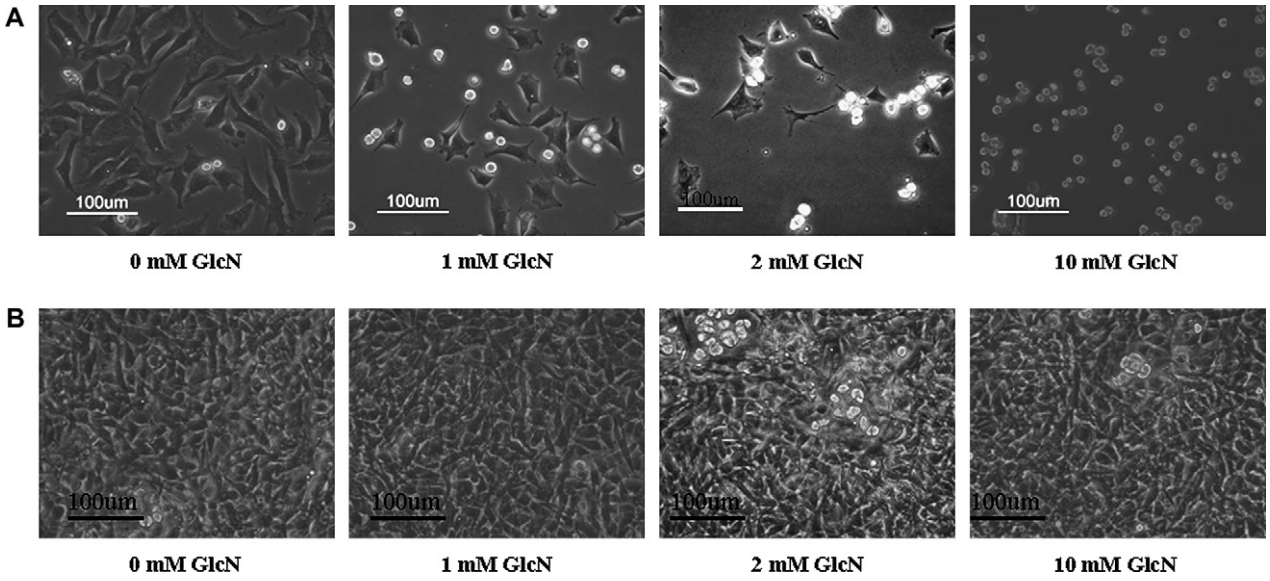

Fig. 1. (A) Cell morphology of chondrocytes exposed to GlcN after 3 days of culture. (B) Cells exposed to GlcN proliferated and achieved comparable confluency to control cultures when they were exposed to GlcN after 6 days of initial plating. Photographs were taken after a total of 11 days of culture.

EFFECT OF GlcN ON GENE EXPRESSION

Cells exposed to 0, 0.7 μM and 2 mM of GlcN expressed genes for cartilage specific markers such as aggrecans and collagen type II (Fig. 4). Using real-time PCR we observed a 1.3- and 4.6-fold increase in aggrecan gene expression when the cells were treated with 0.7 μM and 2 mM GlcN, respectively. A similar increase in collagen type II expression was also observed (1.2- and 1.8-fold increases with 0.7 μM and 2 mM GlcN, respectively). Additionally, GlcN was found to up-regulate TGF-β1 mRNA levels in a dose-dependent manner. In particular, 1.3- and 1.75-fold increases in TGF-β1 mRNA levels were observed with 0.7 μM and 2 mM GlcN, respectively. A similar statistically significant up-regulation of gene expressions was observed for 3D constructs incubated with 2 mM GlcN (data not shown).

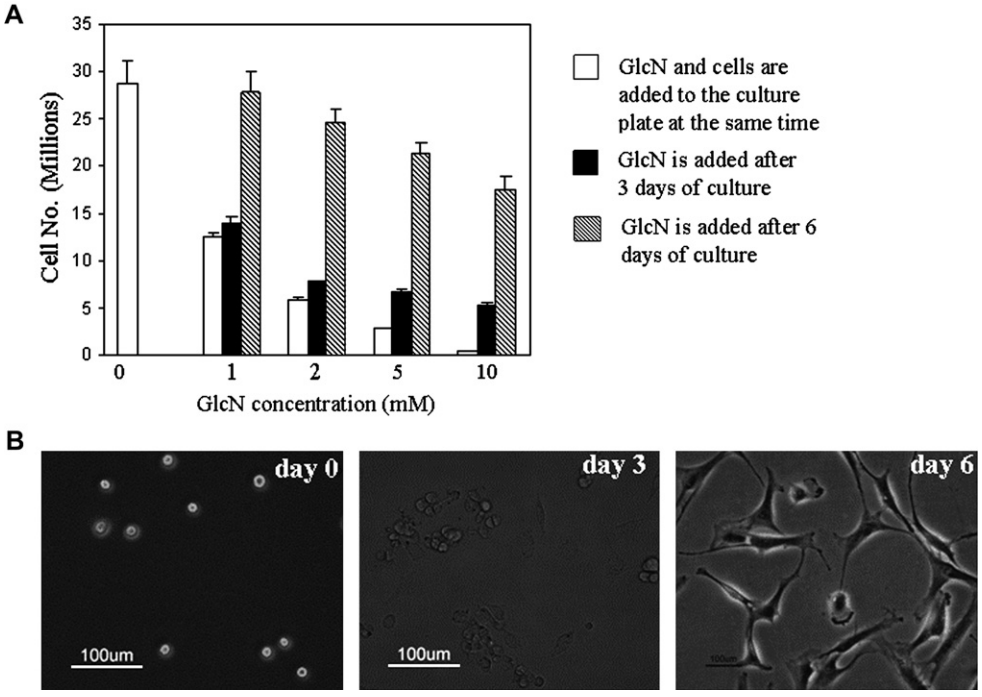

Fig. 2. GlcN tolerance of chondrocytes depends upon when GlcN is added to culture. Proliferation of cells after 11 days in monolayer at various experimental conditions. (B) Snapshots of monolayer cultures obtained via optical microscope at the time of GlcN exposure.

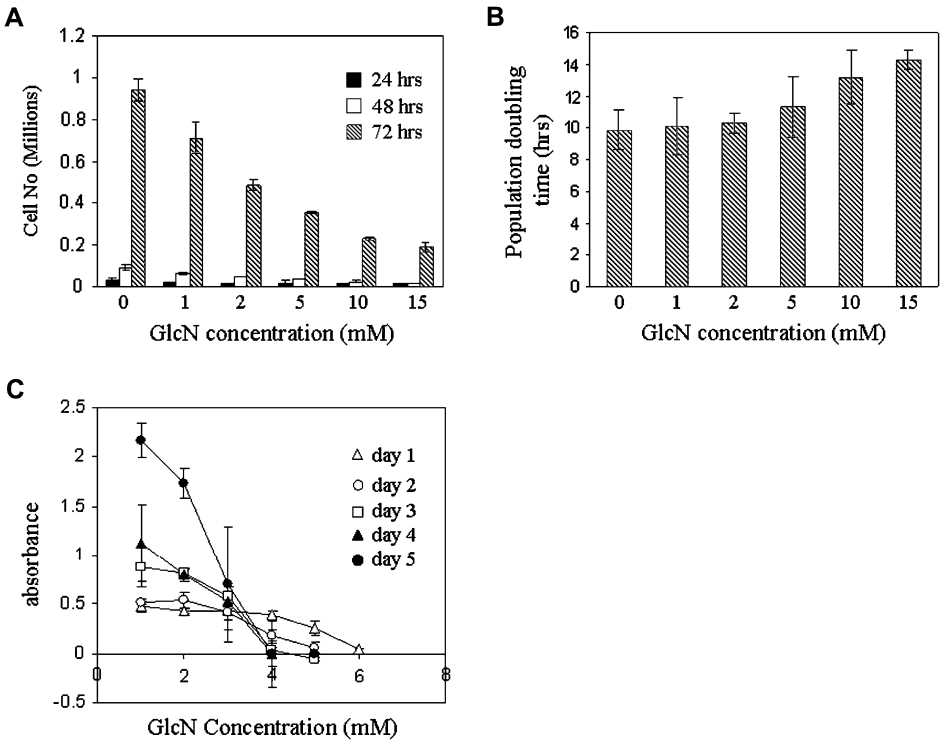

Fig. 3. (A) Growth kinetics of chondrocytes in culture medium containing different concentrations of GlcN. Chondrocytes were plated at a cell density of 5000 cells/cm^2, and exposed to GlcN immediately. Initial population doubling for the first 3 days after plating the cells. Cells were harvested after 24, 48, and 72 h and counted using a coulter counter. (B) Population doubling times. (C) Cell proliferation for 5 days after plating the cells determined by WST-1 assay. Higher absorbance indicates more cells.

EFFECT OF GlcN ON CHONDROCYTES IN 3D HYDROGEL CULTURE SYSTEMS

Three-dimensional culture systems are known to provide enhanced cell biological activities and physiological environment to the encapsulated cells[32,33]. Our earlier studies have shown that PEGDA hydrogels provide a suitable environment to chondrocytes and chondroprogenitor cells[34]. In this study, chondrocytes were successfully encapsulated in 10% (w/v) PEGDA hydrogels at a cell density of approximately 1.5 million cells per construct. Gross visualization of the constructs revealed that chondrocytes were homogenously distributed throughout the hydrogel after photopolymerization. The majority of cells within the hydrogel were viable immediately after encapsulation while some dead cells were sparsely distributed throughout the hydrogel (data not shown).

The 3D constructs were cultured in chondrocyte medium containing 0, 2 and 15 mM GlcN. The cells in 3D constructs remained viable in the presence of the same GlcN concentrations that caused cell death in monolayer cultures. However, as in 2D cultures, a decrease in cell proliferation with increasing GlcN concentration was observed in 3D hydrogels systems [Fig. 5(A)]. Histological analysis (Fig. 6) demonstrates that 3D constructs produced proteoglycans in all three experimental conditions (culture medium with 0, 2 and 15 mM GlcN). Quantitatively, we observed that GAG synthesis was maximized when the GlcN concentration in the culture medium was 2 mM, but at higher concentrations

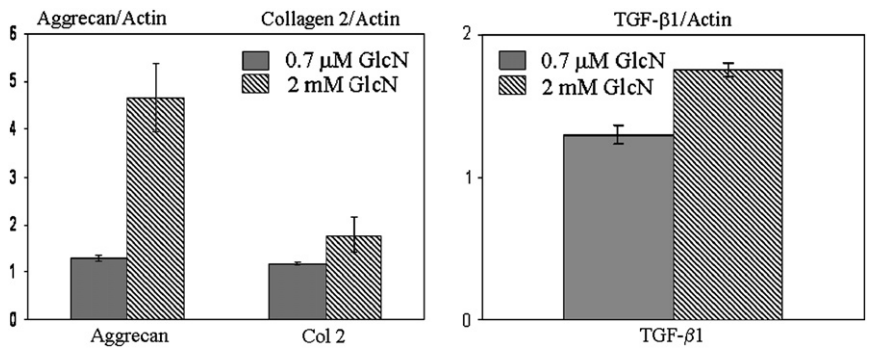

Fig. 4. Real-time PCR of monolayer cultured chondrocytes indicates a dose-dependent up-regulation of (A) cartilage specific markers collagen type II and aggrecan mRNA and (B) TGF-β1 mRNA with GlcN treatment.

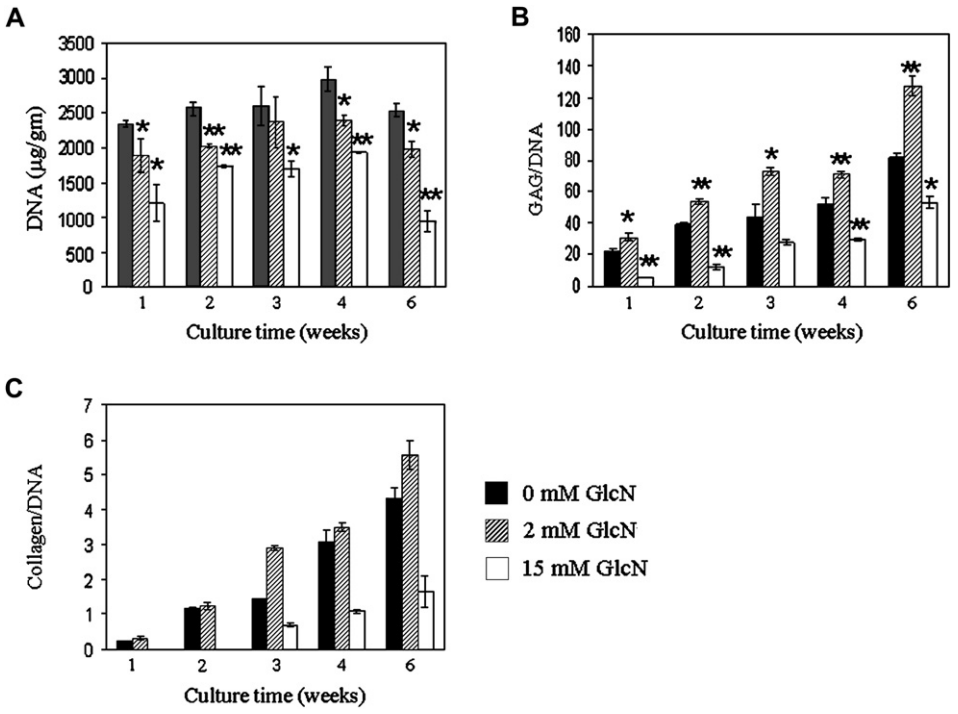

Fig. 5. Biochemical analysis of DNA (cell proliferation), GAG, and collagen ($n = 6$) for 3D constructs cultured with 0, 2, and 15 mM GlcN. (a) Cell proliferation of chondrocytes encapsulated within hydrogel (b) GAG, and (c) collagen content for encapsulated chondrocytes normalized by DNA content (w/w; $n = 6$). $^{*}P < 0.05$ and $^{**}P < 0.007$.

of GlcN (15 mM), GAG matrix production was inhibited [Fig. 5(B)]. A similar trend in the accumulation of collagen type II was observed by immunofluorescent staining (Fig. 6) and collagen assay [Fig. 5(C)]. Type II collagen staining extends radially from the cells to a greater distance with 2 mM GlcN incubation compared to 0 and 15 mM GlcN. No collagen type I protein was detected by immunofluorescent staining which agrees with the PCR analysis. In our 3D studies, the physiological concentration of GlcN (0.7 μM) was found to be too low to produce any significant effect

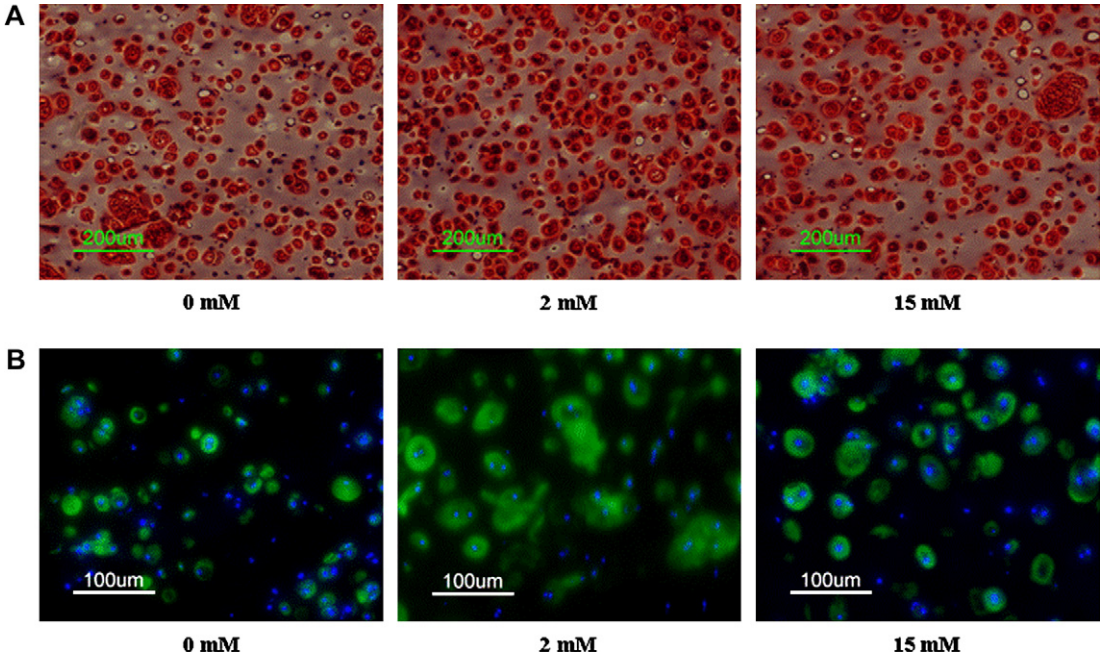

Fig. 6. (A) Safranin-O and, (B) collagen type II staining (nuclei of the cells were stained with DAPI (blue)) for 3D hydrogels constructs cultured in the indicated concentrations of GlcN.

as those observed at 2 mM. Tunnel staining showed an insignificant number of apoptotic cells after GlcN exposure as shown in (Fig. 7).

Discussion

The results described here represent the first comprehensive and systematic investigation of GlcN effect on chondrocytes in 2D and 3D culture systems. Discrete differences in GlcN tolerance were observed between 2D and 3D culture conditions. This may be attributed to the various niches the two conditions provide to the cells. There are a number of differences between 2D monolayer and 3D constructs which include both cellular morphology as well as the microenvironment surrounding the cells. Three-dimensional hydrogels provide a closer *in vivo* cartilage environment to cells by entrapping secreted extracellular matrix (ECM), and maintaining spherical cellular morphologies. From a structural perspective, native AC, which is composed of extracellular matrix components filled with 80% water and 1–10% of chondrocytes, is very similar to the cell-laden hydrogels considered here. Another difference is that the extent of GlcN exposure may also vary between the two culture conditions. In monolayer culture conditions, cells are exposed to bolus dose of GlcN compared to 3D cultures. In the case of 3D constructs, GlcN diffuses into the hydrogel and the diffusion time is highly dependent upon the dimensions of the hydrogels. It has been shown that the characteristic pore sizes of 10% PEGDA hydrogels are on the order of 10 nm[35]. The large pore sizes along with the small hydrogel dimensions (6 mm diameter $\times$ 4 mm length) allow for fast equilibration of monosaccharide concentrations within the hydrogels.

Results from 2D and 3D cultures indicate that exposure to GlcN decreases cell proliferation, which is in agreement with previous studies that show growth inhibition of various cells in the presence of glucose and GlcN[36–38]. The GlcN-induced cell death in monolayer cultures at high GlcN concentrations (10 and 15 mM) under D0 culture conditions is possibly adhesion related. A close examination of these cells shows that they did not adhere to the culture dish, which may eventually lead to cell death. This hypothesis is supported by the observation that cells which were exposed to the same GlcN concentration after given sufficient time to adhere onto the culture dish (i.e., under D3 and D6 conditions) exhibit good cell viability and reach 100% confluency within comparable time frames to that of the control. These observations emphasize that chondrocytes need to be adhered onto the culture dish when they are exposed to an adherent dependent culture condition (monolayer culture) in order to survive at high GlcN concentrations.

Incubation of 3D constructs with 2 mM GlcN–chondrocyte medium resulted in highest cartilage specific matrix production, GAG and collagen type II. However, this effect was only observed when cells were treated with 2 mM or less GlcN concentrations, which suggests that an optimal concentration of GlcN is required for it to have beneficial effects. This finding is in agreement with the previously reported studies, which show no visible stimulatory effect of GlcN on cartilage matrix synthesis at low exogenous GlcN concentrations[25,39]. We found that higher amounts of GlcN in the culture medium had adverse effects on chondrocyte matrix production. Kim and Conrad reported a similar trend in CS accumulation of chick embryo vertebral cartilage cultured in varying GlcN concentrations[39]. The above authors attributed this trend to GlcN-induced inhibition of protein synthesis at high GlcN concentration. It has been documented

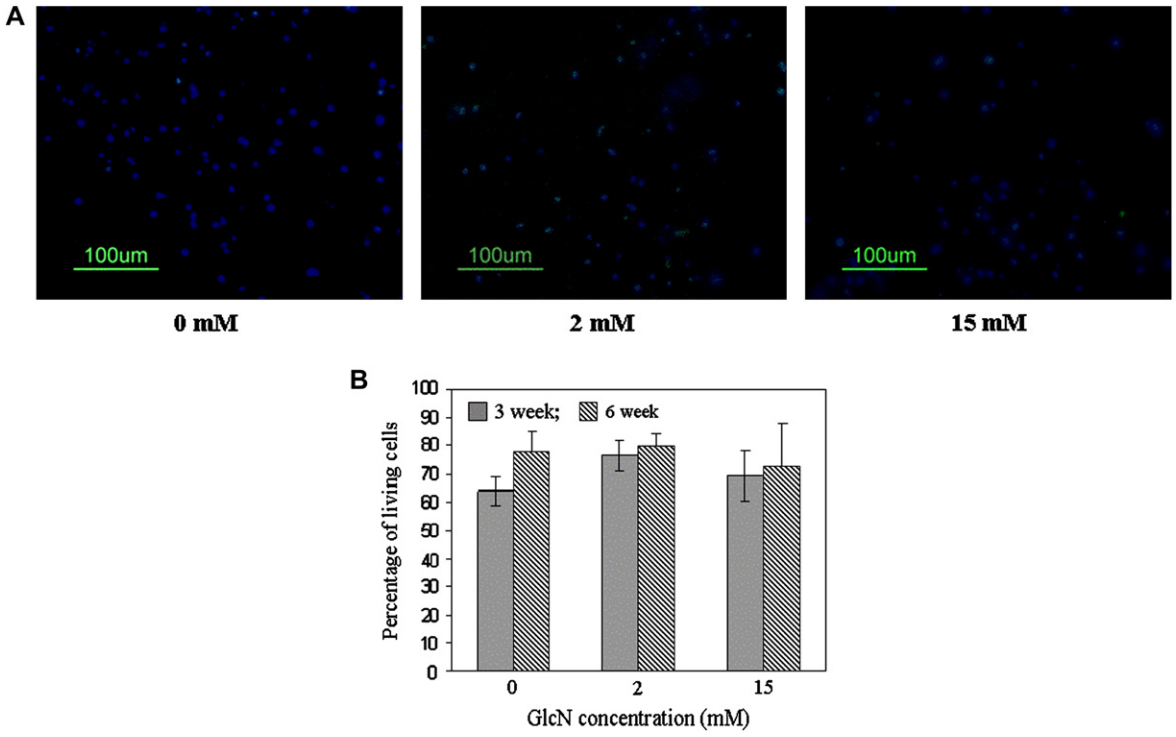

Fig. 7. (A) Tunnel staining for apoptotic cells after incubated in the presence of varying GlcN concentrations. (B) Percentage of living cells after 6 weeks culture in presence of varying GlcN concentrations.

that mucopolysaccharide synthesis of chondrocyte cultures is depressed when protein synthesis was blocked[40]. This optimal 2 mM GlcN concentration which renders beneficial effect on cartilage matrix production is thousand folds higher than the concentration achieved in the synovium after consuming the clinically recommended dose[14,15]. Even though we have observed that 0.7 μM GlcN results in some up-regulation of mRNA levels corresponding to matrix markers, such low concentrations of GlcN do not result in significant matrix production (as seen from 2 mM GlcN). However, various other studies have shown that low levels of GlcN ranging from 20 to 100 μM can inhibit IL-1 stimulated gene expressions (and not higher matrix production), thereby alleviating some of osteoarthritic symptoms[22,41]. It is also important to note that we have used healthy chondrocytes to understand the effects of GlcN and not the osteoarthritic chondrocytes, which may have a different behavior in the presence of GlcN.

One of the motivations for the use of GlcN for treating OA is based on their anti-catabolic effects such as inhibiting the anti-inflammatory responses[21–24,41]. Another rationale for using GlcN stems from the knowledge that GlcN is one of the building blocks of GAG. Therefore it has been hypothesized that exogenously supplied GlcN is directly incorporated into the polysaccharide components (i.e., CS) of the ECM. This hypothesis, however, does not explain the GlcN medicated increase in collagen type II. New studies that disprove the notion of direct incorporation of exogenously supplied GlcN into matrix proteoglycans are beginning to appear[25]. Based on our results, we propose a novel explanation to account for the increased production of extracellular matrix through GlcN mediated up-regulation of TGF-β1. As described before, GlcN was found to up-regulate TGF-β1 mRNA levels in a dose-dependent manner in both 2D and 3D constructs. We therefore believe that GlcN mediated increase in the production of specific matrix components involves TGF-β1 up-regulation, possibly through the hexosamine pathway. Indeed, it has been shown in mesangial cells that exogenously supplied glucose and GlcN enhance the matrix production through an up-regulation in TGF-β1 which was proven to be acting through the hexosamine pathway[36,42,43]. TGF-βs have a long history of beneficial effects in the AC. For instance, this growth factor is known to stimulate the collagen and GAG production of articular chondrocytes[44,45]. TGF-βs also regulate cartilage fracture repair by extracellular matrix production[44]. TGF-β is considered as multifaceted cytokine that plays a key role in many downstream effects such as mesenchymal differentiation, matrix production, preventing de-differentiation and controlled differentiation of stem cells[45,46]. In adults, TGF-βs are also believed to maintain a critical balance between the various anabolic and catabolic functions of chondrocytes for proper functioning of the cartilage[44,47].

Conclusion

The present study provides evidence that prolonged exposure of primary chondrocytes to optimal concentrations of GlcN increases matrix production with concomitant inhibition of chondrocyte proliferation. The effect of GlcN on chondrocytes was found to be strongly dependent upon the culture conditions. Our results indicate the presence of a narrow GlcN concentration range over which the chondrocytes produced the maximum levels of type II collagen, and aggrecans. To the best of our knowledge, the present study is the first of its kind that demonstrates GlcN mediated up-regulation of TGF-β1 in chondrocytes. We believe that by promoting expressions of these extracellular molecules and TGF-β1, optimal amount of GlcN preserves cartilage tissue and promotes its repair upon damage. This study paves way for the development of better clinical strategies for cartilage repair involving localized and controlled release of GlcN into the defect site.

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Supplementary data

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