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Quality assessment of cellular and tissue-based products using liquid chromatography-tandem mass spectrometry

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ABSTRACT

We are currently conducting clinical research on cell sheets for cartilage regeneration. One issue with the future use of chondrocyte sheets as cellular and tissue-based products is quality assessment. Currently, chondrocyte sheets are evaluated using invasive methods that cannot be performed on every sheet produced. We report here on our liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique that allows the noninvasive assessment of every sheet using only 50 μl of culture medium. We found that LC-MS/MS could be used to confirm cell sheet viability through the measurement of glucose and glutamine uptake, to estimate extracellular matrix production by measuring serine consumption, to estimate cell kinetics by measuring cytidine and uracil concentrations, and to estimate melanoma inhibitory activity level by measuring pyridoxal concentration. LC-MS/MS may be useful for the noninvasive assessment of products to be used in regenerative medicine.

1. Introduction

We are currently conducting clinical research on cell sheets for cartilage regeneration [1]. One future issue with the use of chondrocyte sheets [2] as cellular and tissue-based products is quality assessment. Currently, both rabbit and human chondrocyte sheets are evaluated by the same methods, which are all invasive methods that cannot be performed on every sheet produced. However, it is now possible to use liquid chromatography-tandem mass spectrometry (LC-MS/MS), a technology recently developed by Shimadzu Corporation, to assess chondrocyte sheet quality. We have found that LC-MS/MS can be used to perform a noninvasive assessment of every sheet. This paper describes the use of LC-MS/MS to analyze chondrocyte sheet production and to determine the optimal number of cell layers for rabbit chondrocyte sheets. We also describe our experiments to determine whether human chondrocyte sheets show similar results.

2. Materials and methods

All animal experiments were conducted in accordance with "The Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health as well as Tokai University's animal handling code of conduct. Experiments using human chondrocytes were conducted after receiving approval from Tokai University's medical ethics committee. LC-MS/MS analysis was conducted after first using modern methods to assess the histological and biochemical characteristics of previously prepared rabbit chondrocyte sheets laminated with one, three, or six cell layers.

2.1. Preparation of cartilage sheets

2.1.1. Cell collection/cell sheet preparation

Chondrocytes and synovial cells were collected from both knees of 10 female Japanese white rabbits (16–18 weeks old, 3 kg average

Abbreviations: LC-MS/MS, Liquid Chromatography-tandem mass spectrometry; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; HE, hematoxylin and eosin; Col, collagen; AGCN, aggrecan; MIA, melanoma inhibitory activity; TGF-β1, transforming growth factor-β1.

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body weight; Tokyo Laboratory Animals Science Co., Tokyo, Japan) for use in rabbit chondrocyte sheets. Chondrocytes and synovial cells for human cartilage sheets were collected from patients aged 20–80 years who underwent surgery at Tokai University; three of the patients underwent total knee arthroplasty surgery, and one patient underwent surgery for a traumatic knee injury.

Collagenase type 1 (Worthington, Lakewood, NJ, USA) was dissolved in Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Gibco, Grand Island, NY, USA) and agitated for 4 h (37 °C, 5% CO_2, 20% O_2). The culture medium was filtered after agitation using a 100-μm cell strainer (BD Falcon Labware, Franklin Lakes, NJ, USA). Synovial cells were cultured in DMEM/F12 with 10% fetal bovine serum (FBS; Gibco) and 1% antibiotic–antimycotic mixture (Gibco), and passaged until P1. Additionally, on culture day 4, ascorbic acid (Wako Junyaku Kogyo, Osaka, Japan) was added to a final concentration of 0.1% in the culture medium (37 °C, 5% CO_2). Human synovial cells were cultured by following the same protocol, except with 20% FBS (Ausgenex, Oxenford, Australia).

After isolating chondrocytes using collagenase, the cells were cryopreserved. Synovial cells were thawed directly before use. When both cell types were ready, they were cocultured. Chondrocytes were cultured on temperature-responsive cell culture inserts (4.2 cm^2; CellSeed Inc., Tokyo, Japan), and synovial cells were seeded on a companion plate (9.6 cm^2; BD Falcon). Temperature-responsive cell culture inserts (UpCell; CellSeed) were made using poly(N-isopropylacrylamide), which undergoes a temperature-dependent and reversible transition from being hydrophobic to hydrophilic, which changes the surface properties of the insert in response to a change in temperature [3] Use of these cell culture inserts allows the removal of cells and the extracellular matrix (ECM) in sheet form by simply adjusting the temperature without the need for enzymes. Cells were cocultured for 14 days at a seeding density of 50000 cells/cm^2 for chondrocytes and 10000 cells/cm^2 for synovial cells [4]. We have previously shown that coculturing chondrocytes and synovial cells increases chondrocyte proliferation [5–7].

2.1.2. Preparation of laminated sheets

After 14 days of coculture, the temperature-responsive cell culture plates were placed at 25 °C for 30 min, and the sheets were then collected. The inserts were removed, and the chondrocyte sheets were manually laminated using polyvinylidene fluoride rings. Rabbit chondrocyte sheets were made by laminating with one, three, or six layers, and human chondrocyte sheets were made by laminating with three layers. After lamination, rabbit chondrocyte sheets were cultured in a cell culture dish ($\varphi = 10$ cm) in DMEM/F12 with 10% FBS, 1% antibiotic–antimycotic mixture, and 0.1% ascorbic acid. Human chondrocyte sheets were cultured in the same way using DMEM/F12 with 20% FBS, 1% antibiotic–antimycotic mixture, and 0.1% ascorbic acid. The culture medium was replaced every 72 h, and the cells were cultured for 7 days.

2.2. Assessment of chondrocyte sheets

Rabbit chondrocyte sheets were assessed by immunostaining. The component cells were counted, and the concentrations of humoral factors were assessed by enzyme-linked immunosorbent assay (ELISA) and LC-MS/MS. For human chondrocyte sheets, the component cells were counted, and the concentrations of humoral factors were assessed by ELISA and LC-MS/MS.

2.2.1. Immunohistological assessment

Rabbit chondrocyte sheets were cultured for 7 days after lamination, embedded using OTC compound 4583 (Sakura Finetechnical Co. Ltd. Tokyo, Japan), and sliced into 0.8-μm-thick cryosections at −80 °C. The tissue sections were stained with 0.1% safranin O and hematoxylin and eosin (HE) dye ($n = 3$). Immunostaining was then performed using antibodies to type I collagen (Col I), type II collagen (Col II), and aggrecan (AGCN). Goat IgG (200 μg/ml), goat anti-human Col I (400 μg/ml), and goat anti-human AGCN (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used for Col I and AGCN, respectively. Mouse IgG1 (100 μg/ml) and anti-Col II mIgG1 (Daiichi Fine Chemical Co. Ltd., Toyama, Japan) were used for Col II ($n = 3$). The sections were then washed with phosphate-buffered saline and stained with biotinylated polyclonal anti-mouse immunoglobulin (1/600; Dako, Glostrup, Denmark). The other sections were incubated with PBS instead of specific primary antibodies and stained as a negative control.

2.2.2. Cell counting

Whole laminated rabbit chondrocyte sheets were dissolved in 10% FBS and collagenase type I (37 °C, 1 h). The cells were counted after counterstaining with trypan blue (Thermo Fisher Scientific, Waltham, MA, USA). Cells were counted on days 0, 3, and 7 after lamination. Measurements on day 0 were conducted by measuring single-layer sheets, and multiplying the results by 3 or 6 instead of measuring the other groups ($n = 3$). Human chondrocyte sheets were dissolved on day 7 after lamination using TrypLE Express (Gibco, 37 °C, 15 min) and collagenase P (Roche, Basel, Switzerland) for 30 min at 37 °C, and then counterstained following the same protocol as that for rabbit chondrocyte sheets for cell counting ($n = 4$).

2.2.3. ELISA

After 7 days of culture after lamination, DMEM/F12 was prepared with 1% FBS and 1% antibiotic–antimycotic, and 3 ml of this medium was added to the cell culture plates. The sheets were transferred to the plates and cultured for 72 h, and the concentrations of melanoma inhibitory activity (MIA) and transforming growth factor-β1 (TGF-β1) secreted into the culture media were measured using ELISA. MIA concentration was measured using a 96-well plate ELISA kit (Roche, Mannheim, Germany), and TGF-β1 concentration was measured using a Quantikine Human TGF-β1 ELISA Kit (R&D Systems, Minneapolis, MN, USA) (rabbit: $n = 3$ each; human: $n = 4$ each).

2.2.4. LC-MS/MS analysis

LC-MS/MS was performed using only 50 μl of the cell culture supernatant. A High-performance liquid chromatography systems (Nexera X2, LCMS-8050 and LC/MS/MS Method Package for Cell Culture Profiling; Shimadzu Corp, Kyoto, Japan) were used, and the data were analyzed using the LabSolutions LCMS. The results are shown as the area ratio between the 2-isopropylmalic acid reference standard and the target molecules. During the culture of the laminated sheets, the culture medium was replaced after 72 h (on day 3) and 144 h (on day 6), when the replaced culture medium was collected for LC-MS/MS analysis from each sample (rabbit: $n = 3$; human $n = 4$). Additionally, DMEM/F12 with 10% FBS, 1% antibiotic–antimycotic mixture, and 0.1% ascorbic acid was measured as a blank sample. Similarly, for human chondrocyte sheets, DMEM/F12 with 20% FBS, 1% antibiotic–antimycotic mixture, and 0.1% ascorbic acid was measured as a blank. For clarity, the data are shown after subtracting the value for the relevant blank sample.

2.3. Statistical analysis

ELISA results were analyzed using a four-parameter logistic curve. All other analyses were performed using EZR statistical

software (Easy R; Saitama, Japan) [8]. One-way ANOVA was used to compare the rabbit chondrocyte sheets between groups. Pearson's product-moment correlation coefficient was used to analyze the relationships between different components detected by LC-MS/MS.

3. Results

As a preface to the experiments using rabbit chondrocytes, we note that they naturally proliferate and repeatedly form laminated layers on their own, whereas human chondrocytes do not exhibit this behavior.

3.1. Tissue thickness

Rabbit chondrocyte sheets were assessed in HE-stained samples. Tissue thickness was calculated as the sum of the sheet thicknesses. Three-layer sheets were the thickest. The layers in the six-layer sheets did not adhere to each other and were much thinner than the cell layer in the single-layer sheet. The thicknesses were as follows: single-layer sheet = 51.9 ± 7.0 μm, three-layer sheet = 116.6 ± 6.5 μm, six-layer sheet = 81.7 ± 9.4 μm.

3.2. Immunohistological analysis

Rabbit chondrocyte sheets were stained with safranin O for assessment. Single-layer sheets stained most strongly (Fig. 1A), but three-layer and six-layer sheets also stained positively (Fig. 1B and C). All sheets stained negatively for Col I (Fig. 1D–G) but positively for Col II (Fig. 1H–K) and AGCN (Fig. 1L–O). There was no clear difference in staining pattern between the sheets with different numbers of layers.

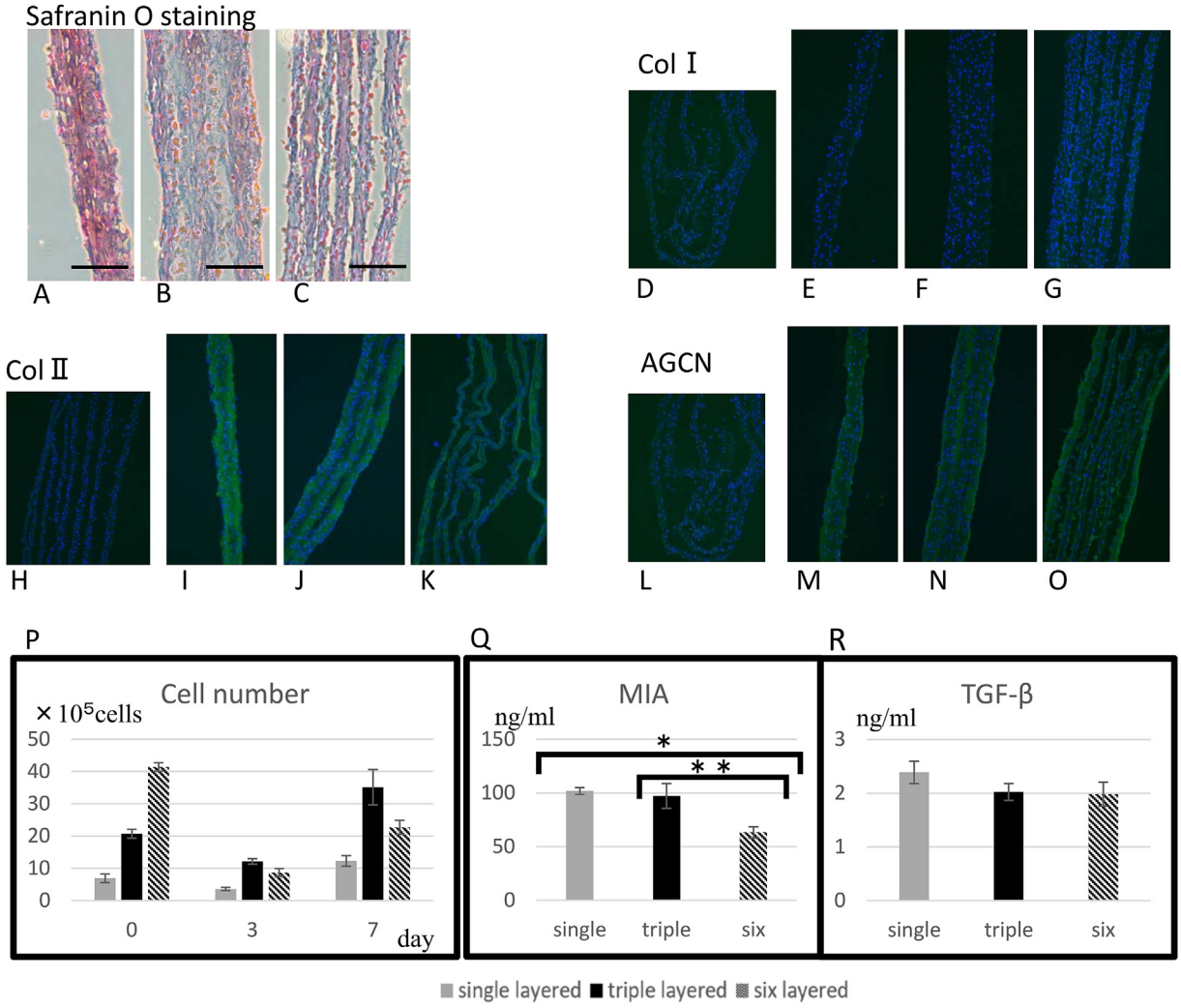

Fig. 1. Histological and biochemical results of the invasive examination. (A–C) Results of safranin O staining. A, B, and C correspond to single-, three-, and six-layer chondrocyte sheets, respectively. Single-layer sheets stained strongly for safranin O; three- and six-layer sheets stained positively but not as strongly. The line represents 50 μm. (D–G) Results of collagen I (Col I) immunostaining. D, E, F, and G correspond to control, single-, three-, and six-layer chondrocyte sheets, respectively. All sheets stained negatively for Col I. (H–K) Results of Col II immunostaining. H, I, J, and K correspond to control, single-, three-, and six-layer chondrocyte sheets, respectively. All sheets stained positively for Col II. (L–O) Results of aggrecan (AGCN) immunostaining. L, M, N, and O correspond to control, single-, three-, and six-layer chondrocyte sheets, respectively. All sheets stained positively for AGCN. (P) Cell number ($\times 10^5$ cells). Day 0: single-layer = 6.9 ± 1.3, three-layer = 20.7 ± 1.3, and six-layer = 41.4 ± 1.3. Day 3: single-layer = 3.6 ± 0.5, three-layer = 12.1 ± 0.8, and six-layer = 7.7 ± 1.2. Day 7: single-layer = 12.3 ± 1.6, three-layer = 35.1 ± 5.5, and six-layer = 22.7 ± 2.2. The vertical axis represents cell count ($\times 10^{-5}$), and the horizontal axis represents days. (Q) Whole-sheet MIA production (ng/ml): single-layer = 101.92 ± 3.19, three-layer = 97.36 ± 11.63, and six-layer = 63.32 ± 5.19. (R) TGF-β1 production (ng/ml): single-layer = 2.39 ± 0.21, three-layer = 2.03 ± 0.16, and six-layer = 1.99 ± 0.22. The vertical axis represents concentration (ng/ml). MIA concentration differed significantly between single-layer and six-layer chondrocyte sheets and between three-layer and six-layer chondrocyte sheets.

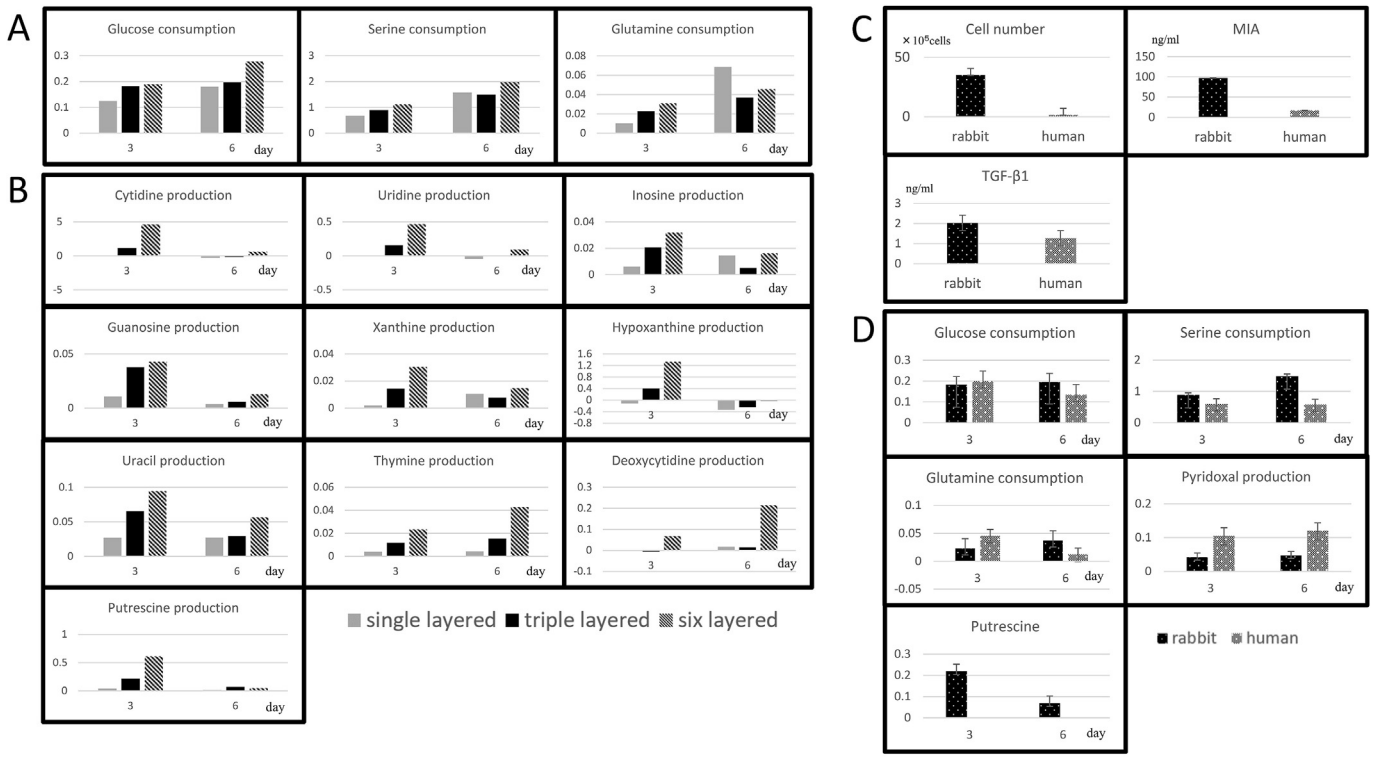

Fig. 2. Results of the cell culture profiling system. (A) Glucose, serine, and glutamine used in all groups. The vertical axis represents the area ratio, which was calculated as the target substance area value divided by the standard substance area value. The horizontal axis represents days. (B) The concentrations of these substances differed by >30% between the three groups. In the six-layer sheets, these concentrations were high on day 3 and low on day 6. Thymine and deoxycytidine were exceptions, and their concentrations were low on day 3 and high on day 6. The vertical axis represents the area ratio, calculated as described above. The horizontal axis represents days. (C) Comparison between rabbit and human chondrocyte sheets. All results are shown for three-layer rabbit and human chondrocyte sheets on day 7. The vertical axis represents units. (D) Comparison of three-layer rabbit and human sheets. The peaks for glucose, serine, and glutamine concentrations occurred on different days in the rabbit and human. The vertical axis represents the area ratio, which was calculated as described in Fig. 1. The horizontal axis represents days.

3.3. Cell counts

The cell count initially decreased and then increased in every group. The three-layer sheets had the highest final cell count, whereas the six-layer sheet exhibited the largest change over time (Fig. 1P). The cell count for human chondrocyte sheets was 1.60 ± 0.22 ($\times 10^5$ cells). The cell counts were much higher in sheets made from rabbit chondrocytes than in those made from human chondrocytes (Fig. 2C).

3.4. Humoral factor production by chondrocyte sheets

For rabbit chondrocyte sheets, MIA secretion differed between single-layer and six-layer sheets and between three-layer and six-layer sheets ($P < .05$ for both comparisons; Fig. 1Q). However, TGF-β1 secretion did not differ significantly between the number of layers (Fig. 1R). The concentrations produced by human chondrocyte sheets were: MIA = 16.72 ± 5.54 ng/ml and TGF-β1 = 1.27 ± 0.31 ng/ml. MIA and TGF-β1 concentrations were higher in rabbit chondrocyte sheets than in human chondrocyte sheets (Fig. 2C).

3.5. Mass spectrometry results

LC-MS/MS detected 95 distinct molecules (Table 1). Of these, 58 molecules differed between the samples and the blank sample. The molecules that exhibited large changes were examined and are marked with an asterisk in Table 1. Glucose, pyruvic acid, isocitric acid, succinic acid, serine, and glutamine were consumed in large quantities by the chondrocyte sheets. Lactic acid, citric acid, fumaric acid, and malic acid were produced in large quantities by the chondrocyte sheets. Most of these substances are closely related to energy production by glycolysis and the citric acid cycle.

There were large differences in the concentrations of the nucleic acid-related molecules cytidine, uridine, inosine, guanosine, xanthine, hypoxanthine, uracil, thymine, deoxycytidine, and putrescine (an amine) between the experimental groups (Fig. 2B). The molecules that were highly active in human chondrocyte sheets were roughly the same as those in rabbit chondrocyte sheets. However, putrescine activity was higher in rabbit than in human chondrocyte sheets ($P < .05$) (Fig. 2D).

4. Discussion

Multiple mechanisms contribute to cartilage tissue repair/regeneration by cell sheets, including tissue repair by cell sheet-derived free chondrocytes, supply of growth factors from the cell sheet, and the role of the cell sheet as a barrier that inhibits the penetration of catabolic factors [9]. Histological analysis showed that every sheet stained successfully with safranin O, and immunostaining showed that all sheets stained positively for Col II and AGCN. However, the three-layer sheets were the thickest and had the highest cell count. All sheets exhibited a transient decrease in cell count on day 3 and an increase by day 7. This transient decrease in cell count may reflect the change in the cell culture environment from the temperature-responsive cell culture insert to a standard culture dish or handling during the laminating process. Nevertheless, rabbit chondrocytes naturally proliferate and repeatedly form

Table 1
The 95 substances identified by the LS-MS/MS method. Substances identified in the cell culture profiling system. The substances marked with (*) had significant changes with blank.

Internal Standard	***Amino Acid and Derivatives***	***Vitamins***
2-Isopropylmalic	2-Aminoadipic acid	4-Aminobenzoic acid
Sugars	4-Aminobutyric acid	Ascorbic acid
Gluconic acid	4-Hydroxyproline	Ascorbic acid 2-phosphate
Glucosamine	5-Glutamylcysteine	Biotin
Glucose*	5-Oxoproline	Choline
Sucrose	Alanine	Cyanocobalamin
Threonic acid	Alanyl-glutamine	Ergocalciferol
Nucleic acid associated compounds	Arginine	Folic acid
Adenine	Asparagine	Folinic acid
Adenosine	Aspartic acid	Lipoic acid
Adenosine monophosphate	Citrulline	Niacinamide
Cytidine*	Cystathionine	Nicotinic acid
Cytidine monophosphate	Cysteine	Pantothenic acid
Deoxycytidine*	Cystine	Pyridoxal*
Guanine	Glutamic acid*	Pyridoxine
Guanosine	Glutamine*	Riboavin
Guanosine monophosphate	Glutathione	Tocopherol acetate
Hypoxanthine	Glycine	***Others***
Inosine*	Glycyl-glutamine	2-Aminoethanol
Thymidine	Histidine	2-Ketoisovaleric acid
Thymine*	Isoleucine	3-Methyl-2-oxovaleric acid
Uracil*	Kynurenine	4-Hydroxyphenyllactic acid
Uric acid	Leucine	Citric acid*
Uridine*	Lysine	Ethylenediamine
Xanthine*	Methionine	Fumaric acid*
Xanthosine	Methionine sulfoxide	Glyceric acid
Antibiotics	N-Acetylaspartic acid	Histamine
Penicilin G	N-Acetylcysteine	Isocitric acid*
	Ornithine	Lactic acid*
	Oxidized glutathione	Malic acid*
	Phenylalanine	O-Phosphoethanolamine
	Pipecolic acid	Putrescine*
	Proline	Pyruvic acid*
	Serine*	Succinic acid*
	Threonine	
	Tryptophan	
	Tyrosine	
	Valine	

multiple layers on their own, which may have contributed to the increase in cell count after the initial decrease.

Our results suggest the biochemical importance of MIA and TGF-β1 in chondrocyte sheets. MIA is a soluble protein that is secreted by malignant melanoma cells and chondrocytes [10]. Humoral MIA level is related to cartilage remodeling and can be used as an objective marker of cartilage characteristics [10]. Stimulating chondrocytes with TGF-β1 increases ECM production, which is related to the maintenance of chondrocyte homeostasis and cell division [11]. The ELISA showed that the concentration of MIA secreted by the sheets was significantly higher for the single-layer and three-layer sheets than for the six-layer sheets. These results indicate that, from a histological and biochemical perspective, three layers is the ideal number of layers for the chondrocyte sheets. The results from the six-layer sheets suggest that increasing numbers of layers limits the diffusion of nutrients through the multiple layers.

LC-MS/MS was developed as a technology to assess undifferentiated cells over time while they are being cultured. Using LC-MS/MS, we detected 95 compounds, including sugars, nucleic acids, proteins, and vitamins. In the discussion below, we focus on two major categories of molecules—those that were consumed or produced at a high level and those whose concentrations differed between experimental groups.

Glucose, glutamine, and serine were all consumed in large quantities (Fig. 2A). Glucose provides a source of energy for all cellular activities, and glutamine is used as a nitrogen source in the biosynthesis of various compounds [12,13]. These two molecules were consumed most heavily between days 3 and 6, when the cell count was increasing. The concentrations of these molecules are affected by cellular activity such as cell division. Although serine is not consumed in large quantities in ordinary cells, it exhibited similar dynamics to glucose and glutamine, which is a characteristic response of chondrocytes to culture. Chondrocytes produce large quantities of the ECM, which is made mainly of proteoglycans, of which serine is a major component [14]. This is a likely reason for the increased serine consumption during the period of high cellular activity from days 3–6.

The concentrations of molecules involved in glycolysis and the citric acid cycle also exhibited marked increases and decreases. The increases and decreases in glycolysis- and citric acid cycle-related molecules in the cell culture profiling system are shown in Fig. 3. The concentrations of glucose and pyruvic acid decreased; both of these are involved in glycolysis, and these changes reflect increased glycolysis in the cells. The increased lactic acid and citric acid concentrations may also reflect the increase in glycolysis. Lactic acid concentration correlated significantly with glucose and citric acid consumption ($P < .05$), providing evidence that the increased amount of lactic acid was produced by glycolysis. However, isocitric acid and succinic acid concentrations decreased whereas fumaric acid and malic acid concentrations increased. This is not the expected citric acid cycle response, and it is possible that the activity of the enzyme that catalyzes the conversion from citric acid to isocitric acid was affected by the culture system. This enzyme is an iron sulfur protein called aconitase, which requires sufficient iron to

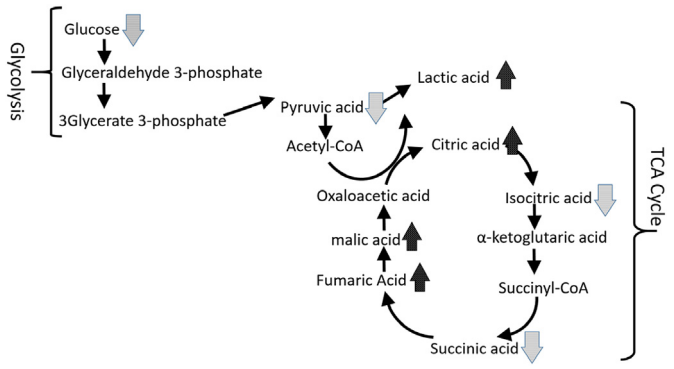

Fig. 3. Glycolysis pathway and citric acid cycle. The arrows correspond to the content for each substance compared with the blank. The data contained in this figure suggest that the citric acid cycle did not operate normally in the chondrocyte sheets.

function properly [12]. Although the FBS used in our research contains iron, it may be useful to monitor iron levels. The low malic acid concentration probably reflected abnormal conversion from citric acid to isocitric acid, and the marked increase in fumaric acid concentration may reflect its presence in the urea cycle [12,13]. Fumaric acid is produced by both the citric acid cycle and the processing of excess NH_4^+ from the breakdown of amino acids. The concentration of fumaric acid measured in our system reflects both the amounts produced by the citric acid cycle and synthesized from excess NH_4^+ from the breakdown of amino acids. This idea is supported by the observation of high fumaric acid and malic acid concentrations on day 3, when there was significant cell death, followed by decreased concentrations by day 6. Our findings suggest that the citric acid cycle was not functioning properly in this culture system.

The articular cartilage is usually a hypoxic environment. Chondrocytes exhibit increased cellular activity in hypoxic conditions with oxygen concentrations of around 2% [15]. These experiments were conducted under 20% oxygen, so the experimental conditions were not necessarily consistent with *in vivo* conditions. Additionally, glycolysis and the citric acid cycle have functions beyond energy production, and other related factors (supplements of amino acids, nucleotides, and so on) should also be considered [16].

We found some molecules, many of which are related to nucleic acids, whose concentrations differed markedly between the experimental groups. The concentrations of these molecules were high on day 3, when there was significant cell death, but decreased by day 6. The concentrations were very high in the six-layer sheets, which had the higher cell death rate, and this finding suggests that these molecules are related to cell death. To compare the changes in concentrations of these molecules with the changes in cell counts, we estimated cell counts for day 6 using the cell count data presented above. Significant correlations between cell count and the changes in the concentrations of cytidine, uridine, guanosine,

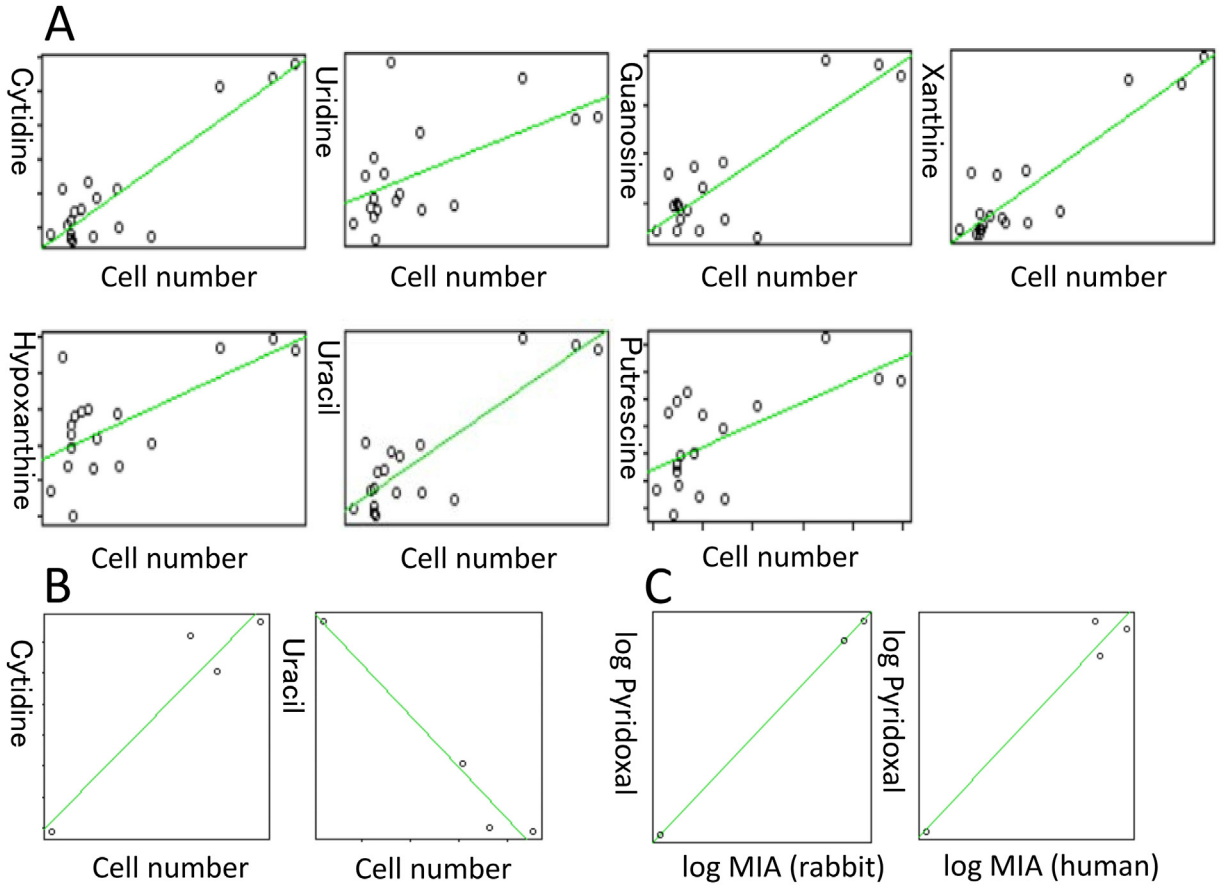

Fig. 4. Correlation between cell count and MIA concentration. (A) Correlations between cell number and concentrations of other nucleic acid substances and putrescine in the rabbit; all correlations were significant ($P < .05$). The vertical axis represents concentration (ng/ml), and the horizontal axis represents cell number ($\times 10^{-5}$). (B) In the human chondrocyte sheets, cytidine and uracil concentrations correlated with cell number ($P < .05$). The vertical axis represents concentration (ng/ml), and the horizontal axis represents cell number ($\times 10^{-5}$). (C) Correlation between MIA and pyridoxal concentrations in both rabbit and human. Log (MIA) and log (pyridoxal) correlated in both the rabbit and human ($P < .05$).

xanthine, hypoxanthine, and uracil were found ($P < .05$) (Fig. 4A). Similar changes were seen in the concentration of putrescine (an amine), and these changes correlated with cell count ($P < .05$). The details of putrescine function are not fully known, but it is involved in amino acid breakdown in living cells and is released from dead cells [17]. In future, these molecules may be useful indicators of large changes in cell count.

It would be ideal to identify molecules related to cytokines, such as MIA and TGF-β1, and to use this to assess the quality of chondrocyte sheets. We were unable to identify any molecules related to TGF-β1, but we did observe that MIA concentration correlated with glucose and pyridoxal concentrations ($P < .05$). At the same time, MIA and pyridoxal concentrations showed a significant logarithmic correlation ($P < .05$). Pyridoxal is one form of vitamin B6 that acts as a prosthetic group for all aminotransferases [12]. To our knowledge, no other studies have reported a relationship between MIA and pyridoxal concentrations in cell culture.

We observed increased glucose, glutamine, and serine consumption in human chondrocyte sheets similar to that observed for rabbit chondrocyte sheets. Cytidine and uracil concentrations correlated with the cell count (Fig. 4 B) ($P < .05$). In addition, a logarithmic relationship between MIA and pyridoxal concentrations was observed (Fig. 4C) ($P < .05$). These results show that LC-MS/MS can be used in the following ways. (1) Glucose and glutamine concentrations can be measured to screen for the biological activity of sheets. (2) Serine concentration can be measured to screen for the presence of the ECM. (3) Cytidine and uracil concentrations can be used to estimate cell kinetics. (4) Pyridoxal concentration can be used to estimate MIA concentration. Further research using human cells is expected to help improve the development of noninvasive methods for assessment of product quality control in regenerative medicine.

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Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.01.001.

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