In vitro differentiation of chondrogenic ATDC5 cells is enhanced by culturing on synthetic hydrogels with various charge densities

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Abstract: We investigated the behavior of chondrogenic ATDC5 cells on synthetic polymer gels with various charge densities: negative charged poly (2-acrylamido-2-methyl-1-propanesulfonic acid) gel, neutral poly (dimethylacrylamide) gel, and copolymer gels of 2-acrylamido-2-methyl-1-propanesulfonic acid and dimethylacrylamide with different compositions (molar fraction of 2-acrylamido-2-methyl-1-propanesulfonic acid, F=0.25, 0.5, 0.75). In insulin-free maintenance medium, the ATDC5 cells cultured on the highly negative charged gels; poly (2-acrylamido-2-methyl-1-propanesulfonic acid) gel and the copolymer gels of 2-acrylamido-2-methyl-1-propanesulfonic acid and dimethylacrylamide (F=0.75), spread and became confluent at day 7, and interestingly formed nodules at day 14, expressing type II collagen and proteoglycan. This result demonstrates that the highly negative charged gels can induce chondrogenic differentiation of ATDC5 cells even in insulin-free maintenance medium, in which the ATDC5 cells cultured on the standard polystyrene dish cannot differentiate into chondrocytes. In insulin-supplemented differentiation medium, ATDC5 cells cultured on the poly (dimethylacrylamide) gel made focal adhesions, rapidly aggregated, and formed large nodules within 7 days, expressing significantly greater levels of type II collagen and proteoglycan than cells cultured on the polystyrene dish and the negative charged gels. These results showed that the neutral gel accelerated chondrogenic differentiation of ATDC5 cells cultured in the differentiation medium. We suggest that the highly negative charged poly (2-acrylamido-2-methyl-1-propanesulfonic acid) gel and the neutral poly (dimethylacrylamide) gel are interesting biomaterials for cartilage tissue engineering as a scaffold with the potential to induce chondrogenic differentiation.

Keywords: ATDC5; Chondrogenic differentiation; Poly (2-acrylamido-2-methyl-1-propanesulfonic acid) (PAMPS); Poly (dimethylacrylamide) (PDMAAm); Copolymer gels of 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) and dimethylacrylamide (DMAAm) (P(AMPS-co-DMAAm) gels); Type II collagen; Aggrecan.

1. Introduction

Articular (hyaline) cartilage tissue cannot spontaneously regenerate *in vivo* and articular cartilage defects are a significant and increasing health care concern [1,2]. In medicine, the strategy of filling osteochondral defects with tissue-engineered cartilage tissue or cell-seeded scaffold material by implantation surgery has become prevalent [3-6], and the induction of chondrogenic differentiation is one of the most important foci in cartilage tissue engineering. To induce chondrogenic differentiation, much basic research has focused on the use of various growth factors and cytokines [7]. However, little attention has been paid to biomaterials that can induce the differentiation of chondrogenic cells.

Hydrogels, because of their three-dimensional network structure and viscoelasticity, which are similar to the macromolecular-based extracellular matrix in biological tissues, are used as scaffolds for regenerating a wide variety of tissues and organs [8,9]. Among various hydrogels, negatively charged polymer gels have shown considerable potential as cell scaffolds, actuators with biomimetic movement, and for replacement of biological tissues [10]. It has been reported that negatively charged polyelectroryte gels have potential in cell scaffolds [11], drug delivery system [12], and microfluidic system [13]. We have found that endothelial cells can spread, proliferate, and reach confluence on synthetic negative charged hydrogels without modification of adhesive proteins, and adhesion, attachment and proliferation of the cells depends strongly on the charge density [11, 14, 15]. This result shows that negative charged hydrogels have potential in using as blood vessel. Moreover, we recently reported that negatively charged polymer based double network gel effectively induces cartilage regeneration in vivo rabbit model [16]. Actually, proteoglycans, primary components of

extracellular matrix in cartilage, are negatively charged polymers, and have been known to induce proliferation and differentiation of chondrocytes [17]. However, it was not elucidated clearly how the negative charge of hydrogel influences the behavior of chondrogenic cells.

Therefore, we examined the behavior of chondrogenic cells on hydrogels with various electrostatic charge densities. We used poly-(2-Acrylamido-2-methyl-1-propanesulfonic acid) (PAMPS) gel, which is negatively charged, and poly-(dimethylacrylamide) (PDMAAm) gel, which is electrically neutral, and copolymer gels of 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) and dimethylacrylamide (DMAAm) (P(AMPS-co-DMAAm) gels) with different compositions. Toward the end of examining the effect of hydrogels on chondrogenic differentiation, we have used murine chondrogenic ATDC5 cells in this study. ATDC5 cells that represent a chondroprogenitor clone can be induced through an insulin-dependent pathway into chondrogenic differentiation that closely reduplicates the cartilage development in vivo [18]. The purpose of this study was to clarify the differentiation behavior of ATDC5 cells when cultured on the PAMPS, PDMAAm, and P(AMPS-co-DMAAm) gels, not only in standard insulin-supplemented medium but also in insulin-free medium.

2. Materials and methods

2.1. Hydrogel preparation

2-Acrylamido-2-methyl-1-propanesulfonic acid (AMPS; Tokyo Kasei Kogyo), dimethylacrylamide (DMAAm; Junsei Chemicals), and 2-oxoglutaric acid (Wako Pure Chemicals, Japan) were used as purchased. N, N'-methylene-bis-acrylamide (MBAA;

Tokyo Kasei Kogyo) was purified by recrystallization from ethanol. Poly dimethylacrylamide (PDMAAm) gel, poly(2-acrylamido-2-methyl-1-propanesulfonic acid) (PAMPS) gel, and copolymer gels of AMPS and DMAAm (P(AMPS-co-DMAAm) gels) were synthesized by radical polymerization. An aqueous solution of 1 mol/l monomer, 4 mol% of MBAA as crosslinker and 0.1 mol% 2-oxoglutaric acid as initiator were prepared in a reaction cell that had been purged with nitrogen gas for 30 min and irradiated with UV light for 10 h. P(AMPS-co-DMAAm) gels were quantitatively tuned by adjusting the molar fraction (F) of AMPS (F=0.25, 0.5, 0.75).

All gel reactions were carried out between a pair of glass substrates, separated by a 1.0 mm thick spacer. After gelation, gels were immersed in 4-(-2-hydroxyethyl)-piperazine-1-ethansulfonic acid (HEPES; Sigma) buffer solution ($NaHCO_3$ 1.55 × 10^{-2} M, HEPES 5 × 10^{-3} M, NaCl 0.14 M, pH 7.4) and the HEPES solution was changed twice every day for 1 week to reach equilibrium.

After sterilizing by autoclaving (120 °C, 20 min), gel disks were punched out of gel plate with a hole punch with a diameter of 1.5 cm. The thickness of gel was about 2–3 mm. The gel disks were then placed in 24-well polystyrene tissue culture dishes.

2.2. Cell culture

The ATDC5 cell line was obtained from the RIKEN cell bank (Tsukuba). The cells were cultured in maintenance medium consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (Invitrogen) supplemented with 5% fetal bovine serum, 10 mg/ml human transferrin (Roche Molecular Biochemicals), and 3 × 10^{-8} M sodium selenite (Sigma-Aldrich) in polystyrene dishes at 37 °C under 5% CO_2.

The differentiation medium to induce chondrocyte differentiation of the ATDC5 cells was made by supplementing the maintenance medium with 10μg/ml bovine insulin (Sigma-Aldrich). In cell culture, the maintenance medium or the differentiation medium were changed twice each week without damaging the gels.

2.3. Study design

The first study was conducted to compare the effects of the gel surface on the fundamental behavior of ATDC5 cells cultured on the PAMPS, PDMAAm gels, and P(AMPS-co-DMAAm) gels in maintenance medium. ATDC5 cells were seeded at 1×10^5 cells/cm^2 on the gel surfaces, and cultured in maintenance medium. Twenty-four hours after seeding, we quantified the number of viable cells on the gels by measuring the dehydrogenase activity of the viable cells. The maintenance medium was changed twice each week without damaging the gels. The cultured cells were observed by phase contrast microscopy at 7 days and 14 days of culture. We performed real-time PCR analyses for gene expression of chondrocyte differentiation markers in the cultured ATDC5 cells at 7 days and 14 days of culture. At day 14, we examined the cells for newly formed matrix using immunocytochemistry for type II collagen and alcian blue staining to visualize sulfated glycosaminoglycan.

The second study was conducted to compare the effects of the gel surfaces on the chondrocyte differentiation of ATDC5 cells again in insulin-supplemented differentiation medium using the same gels. ATDC5 cells were seeded at 1×10^5 cells/cm^2 on the gel surfaces and cultured in differentiation medium, which was changed twice each week without damaging the gels. The cultured cells were observed by phase contrast microscopy at 3 days and 7 days after seeding. At day 7, we also

performed real-time PCR analyses of gene expression of chondrocyte differentiation markers and examination by immunocytochemistry for type II collagen and alcian blue staining. The 7-day period for the examinations was chosen because we intended to observe the ATDC5 cells' behavior before the day 14 at which the cells cultured on the standard polystyrene dish were known to differentiate into chondrocytes in the differentiation medium.

Statistical analyses were performed using analysis of variance (ANOVA) with Dunnett's test. The significance level was set at $p = 0.05$.

2.4. Evaluation methods

2.4.1. Measurement of cell proliferation on the polymer gels

ATDC5 cells were seeded at 10^5 cells/well on tissue culture polystyrene, PDMAAm gel, P(AMPS-co-DMAAm) (F=0.25, 0.5, 0.75), and PAMPS gel. After indicated time of seeding, the surfaces of polystyrene and gels were rinsed with PBS(-), and then the number of cells attached to the surfaces was counted with a water-soluble tetrazolium salt (WST-8)–based colorimetric microplate assay according to manufacturer's instructions (Cell Counting Kit-8, Dojindo Molecular Technologies, Inc) (19). WST-1 and methoxy-PMS mixtures were poured into each well, and then incubated for 2 hours. The absorbance of the solution in each well was measured at 450 nm using a microplate reader (Wako). We prepared a calibration curve using the data obtained from the wells that contain known numbers of viable cells.

2.4.2. Real-time PCR analysis

Total RNA was isolated from the ATDC5 cells using the RNeasy Mini Kit

(Qiagen) following the manufacturer's protocol. The RNA concentration was determined using a NanoDrop spectrophotometer (NanoDrop Technologies) and reverse transcription reactions were performed from 0.2 μg of total RNA using a cDNA synthesis kit (Takara). Real-time PCR reactions for GAPDH, collagen II, collagen I and aggrecan were conducted using the SYBR green system. Primer sequences are described in Table 1. Real-time PCR reactions were performed using the Thermal Cycler Dice Real Time System (Takara). Samples were held at 95 °C for 10 min, followed by 40 amplification cycles consisting of a denaturation step at 95 °C for 15s, and an extension step at 60 °C for 1 min. The expression level of the gene was normalized to GAPDH.

2.4.3. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline without calcium (PBS(–)), and permeabilized with 0.1% Triton X-100 in PBS, followed by pretreatment to block nonspecific reactions with 5% nonimmune goat serum in PBS(–). For collagen staining, the primary immunoreaction was carried out with a mouse monoclonal antibody against type II collagen (Abcam). The secondary immunoreaction was carried out with Alexa 488-conjugated goat anti-mouse IgG (Invitrogen) in 1% nonimmune goat serum in PBS, followed by rinsing with PBS(–). For cell nucleus staining, cells were incubated on 1 μg/ml Hoechst 33258 (Dojindo) for 1 min, followed by rinsing with PBS(–). Fluorescent images were recorded with a fluorescence microscope (Nikon Eclipse TE 300) and a Sight DC digital camera (Nikon).

2.4.4. Alcian blue staining

To visualize the accumulation of sulfated glycosaminoglycan, cells were rinsed with PBS, fixed with paraformaldehyde for 20 minutes, stained with 1% alcian blue 8GS (Fluka) in 0.1 M HCl for 5 min at room temperature, and rinsed with distilled water. The accumulation of GAGs was assessed microscopically using phase contrast.

3. Results

3.1. Effects of the gel surface on ATDC5 cells cultured in maintenance medium

We have reported that cell adhesion, attachment and proliferation are strongly dependent on charge density of polymer gels [11, 14, 15]. In this study, we investigated cell proliferation of ATDC5 cells on polymer gels with different charge density. By maintaining the total monomer (mixture of AMPS and DMAAm) concentration at 1M and cross-linker (MBAA) concentration at 4 mol%, while varying the molar fraction (F) of AMPS (defined as the molar ratio of the anionic monomer to the total amount of the monomers in the feed) in the monomer mixture, we were able to obtain polymer gels with different charge density; PDMAAm gel, P(AMPS-co-DMAAm) gels (F=0.25, 0.5, 0.75), and PAMPS gel. The number of spreading cells on the polymer gels was quantified with dehydrogenase activity in the cells.

The PDMAAm gel and the P(AMPS-co-DMAAm) gel (F=0.25) had a significantly low degree of attachment and proliferation rate, while the P(AMPS-co-DMAAm) gel (F=0.75) and the PAMPS gel showed significantly high degree of attachment and proliferation rate, being almost the same as in the polystyrene dish, as shown in Figure 1. In addition, our result showed that the cells almost ceased to proliferate at confluence to form monolayer at day 4, and hardly proliferated any more in the polystyrene dish,

while the cells stopped transiently proliferating at day 4 but restarted to proliferate at day 7 in the P(AMPS-co-DMAAm) gel (F=0.75) and the PAMPS gel.

Phase contrast microscopy showed that ATDC5 cells cultured in maintenance medium on polystyrene proliferated to form a confluent monolayer but did not form nodules. Most of the ATDC5 cells cultured on PDMAAm gel did not attach to the gel, and the few cells that attached on the gel remained round and were almost detached from the gel surface by the mechanical stress of the medium changes over the 14 days (Figure 2). ATDC5 cells cultured on the P(AMPS-co-DMAAm) gel (F=0.5) aggregated with spatial heterogeneity but did not form nodules within 14 days (Figure 2). In contrast, on the P(AMPS-co-DMAAm) gel (F=0.75) and the PAMPS gel, cells adhered to the whole surface, spread, and became confluent within 7 days. Interestingly, it was found that after being confluent, some cells aggregated and formed nodules, that are one characteristic of chondrogenic differentiation, within 14 days despite absence of insulin stimulus (Figure 2).

Real-time PCR could only be performed using the cells cultured on the P(AMPS-co-DMAAm) gel (F=0.5, 0.75), the PAMPS gel, and polystyrene, because the cells cultured on the PDMAAm gel and the P(AMPS-co-DMAAm) gel (F=0.25) did not sufficiently attach to the gel surface. After 7 days of culture, there were no significant differences in aggrecan gene expression among the polystyrene dish, P(AMPS-co-DMAAm) gel (F=0.75), and the PAMPS gels, while aggrecan gene expression in cells grown on the P(AMPS-co-DMAAm) gel (F=0.5) was significantly greater than that seen on the other surfaces (Figure 3B). However, after 14 days of culture, aggrecan gene expression in cells grown on the P(AMPS-co-DMAAm) gel (F=0.75) and PAMPS gels was significantly greater than that of cells grown on

polystyrene, whereas aggrecan gene expression in cells grown on the P(AMPS-co-DMAAm) gel (F=0.5) was not significantly different from those grown on polystyrene (Figure 3B). In particular, the cells grown on the PAMPS gel showed the greatest aggrecan gene expression at 14 days after culture. Similar results were obtained with regard to type II collagen gene expression. Namely, at 14 days after culture, type II collagen gene expression in cells grown on the P(AMPS-co-DMAAm) gel (F=0.75) and PAMPS gels was significantly greater than that in cells grown in polystyrene dishes (Figure 3C). With regard to type I collagen, gene expression in cells cultured on P(AMPS-co-DMAAm) gel (F=0.5) and P(AMPS-co-DMAAm) gel (F=0.75) and PAMPS gel was significantly lower than that of cells grown on polystyrene up to 14 days (Figure 3A).

Immunocytochemistry and alcian blue staining showed obvious expression of type II collagen and sulfated glycosaminoglycan by the cells cultured on the P(AMPS-co-DMAAm) gel (F=0.75) and PAMPS gels, and weak expression of type II collagen and sulfated glycosaminoglycan by the cells in the peripheral regions of the aggregated cells growing on the P(AMPS-co-DMAAm) gel (F=0.5) at 14 days after culture (Figure 4). These results showed that ATDC5 cells significantly differentiated into chondrocytes when cultured on P(AMPS-co-DMAAm) gel (F=0.75) and PAMPS gels.

3.2. Effects of the gel surface on the chondrogenic differentiation induced by the insulin-supplemented medium

Phase contrast microscopy showed that ATDC5 cells cultured on polystyrene dishes and the PAMPS gel were widely adherent to the substrate and spread and became

confluent at day 3 after culture, and nodule formation was not found at up to 7 days (Figure 5). In contrast, the cells seeded on the PDMAAm gel formed focal adhesions, rapidly aggregated, and formed large nodules at day 3 (Figure 5). The cells cultured on the P(AMPS-co-DMAAm) gel (F=0.5) partially attached and spread without frequent nodule formation, and partially formed focal adhesions, aggregated, and formed nodules at day 7 (Figure 5). In addition, the cells cultured on polystyrene formed nodules and differentiated into chondrocytes at 14 days after culture (data not shown).

In the real-time PCR analyses performed at 7 days after culture, the expression of both aggrecan and type II collagen genes in cells grown on the PDMAAm gel and P(AMPS-co-DMAAm) gel (F=0.5) were significantly greater than for cells grown on polystyrene, while there were no significant differences between the PAMPS gel and polystyrene (Figure 6B and C). At 7 days after culture, type I collagen gene expression in cells cultured on the PDMAAm gel, P(AMPS-co-DMAAm) gel (F=0.5), and PAMPS gel was significantly lower than that of cells grown on polystyrene (Figure 6A).

The immunocytochemistry and alcian blue staining at 7 days after culture showed that type II collagen and sulfated glycosaminoglycan were greatly expressed in the aggregated cells cultured on the PDMAAm gel, were expressed in places in cells cultured on the P(AMPS-co-DMAAm) gel (F=0.5), and were rarely expressed in cells cultured on the PAMPS gel and polystyrene (Figure 7A and B). These results showed that within 7 days, ATDC5 cells had clearly differentiated into chondrocytes when cultured on PDMAAm gel. These results means that PDMAAm gel accelerated chondrogenic differentiation in comparison to PAMPS gel and the P(AMPS-co-DMAAm) gels.

4. Discussion

The initial study, performed in insulin-free maintenance medium, showed that attachment and proliferation of ATDC5 cells increased with negative charge density. This result is consistent with a previous study using endothelial cells on copolymer gels consist of dimethylacrylamide and sodium salt of 2-acrylamido-2-methyl-1-propanesulfonic acid (NaAMPS), P(NaAMPS-co-DMAAm)[14,15]. Electrostatic interaction between cells and negatively charged gels cannot explain the adhesion force between cells and the gels because the cell surface is also negatively charged. We previously reported that when Zeta potential of the gel was higher than -20 mV (molar ratio of NaAMPS F<0.4), the concentration of adhesion proteins such as fibronectin adsorbed on the gels was low and cell density was also low, whereas when Zeta potential of the gel was decreased to -20 mV (molar ratio of NaAMPS F= 0.4) the concentration of the proteins adsorbed on the gels began to dramatically increase and the cell density also increased [14]. Similarly, our present result showed that attachment and spreading of ATDC5 cells on the gels had very low degree at molar fraction of AMPS, F=0.25, began to dramatically increase at F = 0.5 (Figure 1). It should be noted that P(AMPS-co-DMAAm) gel and P(NaAMPS-co-DMAAm) gel are the same except the counter-ion of the polyelectrolyte. In the buffer solution, P(AMPS-co-DMAAm) gels changes to P(NaAMPS-co-DMAAm) gels, since most of the H^+ dissociated from AMPS in the P(AMPS-co-DMAAm) gel is exchanged to Na^+. This indicates that adhesion proteins from serum adhered to the surface of negatively charged gel with high ratio of AMPS by electrostatic interaction and induced high level of cell attachment, spreading, and proliferation on the gels.

The most noteworthy result in this study was that on the highly negative charged gels such as PAMPS gel and P(AMPS-co-DMAAm) gel (F=0.75), ATDC5 cells continued to proliferate even after confluences, and formed nodules. The cells on the PAMPS gel and P(AMPS-co-DMAAm) gel (F=0.75) also expressed chondrogenc markers such as highly type II collagen and aggrecan within 14 days even in insulin-free maintenance medium, in which ATDC5 cells cultured in standard polystyrene dishes remained undifferentiated. These results demonstrate that the negative charged PAMPS can induce chondrogenic differentiation of ATDC5 cells without growth factors for differentiation. High cell proliferation rate on the highly negative charged gels may result in the required cell density to support cell differentiation. However, induction of chondrogenic differentiation cannot be explained only by high proliferation rate because polystyrene dish with high proliferation rate could not induce chondrogenic differentiation. It is speculated that negative charged PAMPS play an important role in chondrogenic differentiation, independently of that in cell proliferation.

It is well known that proteoglycans, which are a predominant component of cartilage matrix, are negatively charged. For example, heparan sulfate proteoglycans play a critical role in Wnt and Hedgehog signal pathways and perlecan, a heparan sulfate proteoglycan, provides a signal to initiate chondrogenic differentiation [20, 21]. Previous reports also demonstrated that perlecan also functions as a reservoir for the storage and protection of various growth factors such as fibroblast growth factor-2, vascular endothelial growth factor, and transforming growth factor β/bone morphogenetic proteins, and that proteoglycans binding to the perlecan enhance the biological activities of these growth factors [22-27]. In addition, it was shown that hyaluronan, a predominant glycosaminoglycan, is essential for forming cell-to-cell

communications during chondrogenic differentiation [28]. The PAMPS gel has similarities with proteoglycans in its negative charge and sulfonic acid base. Thus the PAMPS gel may play the roles like proteoglycans. for chondrogenic differentiation. The PAMPS gel may directly function as a signal molecule to interact with receptors for signaling pathways. However, from the fact that the main similarity between PAMPS and proteoglycan is their negative charges, it is more likely that PAMPS gel may act as an effective reservoir for signaling molecules or growth factors present in the maintenance medium, leading to enhancement of ATDC5 cell adhesion and chondrogenic differentiation.

However, it should be noted that ratio of AMPS and DMAAm influences not only charge density but also mechanical property of hydrogels because degree of swelling of hydrogels is strongly influenced by charge density of hydrogels [11]. It was reported that the mechanical properties of a material surface significantly affect culture results [29]. Therefore, the physical conditions created by the mechanical properties of the gel surface may affect chondrogenic differentiation. Further studies are needed to elucidate the detailed role of the PAMPS gel in chondrogenic differentiation.

The second study, performed using standard insulin-supplemented differentiation medium, showed that the neutral PDMAAm gel accelerated ATDC5 cell aggregation and differentiation into chondrocytes in comparison with cells grown on polystyrene and PAMPS gel. We should note that the characteristics of the ATDC5 cells differ between the first and second studies. That is, in the second study, the cells had been committed to differentiate into chondrocytes, while in the initial study the cells were not committed to differentiation. The second study indicated that the chondrogenic differentiation process of the ATDC5 cells, as initiated by the insulin-supplemented

medium, is influenced significantly by the physical properties of the material surface onto which the cells are attaching.

According to previous *in vitro* studies, inhibition of spreading of chondrogenic cells on a substratum promotes cell aggregation, resulting in enhancement of chondrogenic differentiation, and micro-mass culture techniques using nonadherent substrata induces chondrogenic differentiation [30,31]. In this study, the PDMAAm gel showed the lowest level of cell attachment. Therefore, we speculate that the weak adhesion of the ATDC5 cells to the PDMAAm gel surface might enhance cell aggregation, and that this aggregation might then enhance differentiation of the ATDC5 cells into chondrocytes. The poor differentiation on polystyrene and PAMPS gel may be due to less aggregation of ATDC5 cells in comparison to PDMAAm gel within 7 days. This result is consistent with previous reports that cell condensation is essential for chondrogenic differentiation [32,33]. In addition, although most of the cells did not attach to the PDMAAm gel and the few cells that attached on the gel remained round in the absence of insulin, some cells attached, aggregated, and then were induced into differentiation on the gel, which indicates that insulin facilitates cell attachment on the gel surface and cell-to-cell adhesion.

Finally, the highly negatively charged PAMPS gel and the neutral PDMAAm gel are interesting biomaterials for cartilage tissue engineering as scaffolds with potential to induce chondrogenic differentiation. We have developed the double-network hydrogel composed of AMPS and DMAAm which has enough mechanical strength to replace cartilage tissue [34, 35]. In addition, we found that the double network gel induces spontaneous cartilage regeneration in vivo rabbit model [16]. However, the molecular mechanism for the double network to induce cartilage regeneration was not elucidated.

In future studies, it remains to be elucidated how the polymer gels induce chondrogenic differentiation in their optimized condition. Further, study with human primary mesenchymal stem cells will be required for application of the polymer gels to human cartilage tissue engineering.

5. Conclusion

With ATDC5 cells cultured in insulin-free maintenance medium, the PAMPS gel and the P(AMPS-co-DMAAm) gel (F=0.75) showed significantly higher attachment and proliferation of cells, which were dependent on the charge density. The ATDC5 cells cultured on these gels spread and became confluent at 7 days after culture, and interestingly formed nodules at 14 days after culture. At 14 days after culture, the type II collagen and aggrecan genes were highly expressed in these cells, and type II collagen and sulfated glycosaminoglycan were present in the matrix. These results demonstrate that the PAMPS gel can induce chondrogenic differentiation of ATDC5 cells even in insulin-free maintenance medium, in which ATDC5 cells cultured in standard polystyrene dishes cannot differentiate into chondrocytes.

When cultured in insulin-supplemented differentiation medium, ATDC5 cells cultured on the PDMAAm gel made focal adhesions, rapidly aggregated, and formed large nodules within 3 days. At 7 days after culture, type II collagen and aggrecan genes were expressed in the cells on the PDMAAm gel to significantly greater degrees than in cells grown on polystyrene, and type II collagen and sulfated glycosaminoglycan were greatly expressed in the matrix. In contrast, ATDC5 cells cultured on polystyrene and PAMPS gel showed wide adhesion, spread, and became confluent within 3 days, but nodule formation was not found within 7 days. Type II collagen and aggrecan genes expression and type II collagen and sulfated glycosaminoglycan deposition were

minimal in cells cultured on PAMPS gel and polystyrene. These results show that the neutral PDMAAm gel accelerated ATDC5 cell aggregation and differentiation into chondrocytes when compared with polystyrene and PAMPS gel.

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Figure Captions

Figure 1. Time course of cell proliferation on various substrata; Polystryrene dish(PS); ···□···· , PDMAAm gel; —×— , P(AMPS-co-DMAAm) (F=0.25); —○— , P(AMPS-co-DMAAm) (F=0.5); -·◆·- , P(AMPS-co-DMAAm) (F=0.75); —▲- , and PAMPS gel; —●— . After incubation, cell attachment and spreading was quantified by the activity of dehydrogenase activity in the cells at indicated time using a Cell Counting Kit (Dojindo, Japan). Values are the mean ± S.D. obtained in four experiments.

Figure 2. Phase contrast microscopy images of ATDC5 cells cultured on various substrata; Polystryrene dish (PS), PDMAAm gel, P(AMPS-co-DMAAm) (molar fraction of AMPS, F=0.5, 0.75), and PAMPS gel, in the absence of insulin at 7 and 14 days of culture. Scale bar, 100 μm.

Figure 3. Gene expression analysis of type I collagen (A), aggrecan (B), and type II collagen (C) in ATDC5 cells cultured on various substrata; Polystryrene dish (PS), PDMAAm gel, P(AMPS-co-DMAAm) (molar fraction of AMPS, F=0.5, 0.75), and PAMPS gel, in the absence of insulin at day 7 (gray bars) and day 14 (black bars). Expression of each gene was measured by quantitative real-time PCR and normalized to GAPDH expression levels. Values are the mean ± S.D. obtained in four experiments. *$p < 0.05$, and ** $p < 0.01$versus polystyrene, by Dunnett's test.

Figure 4. Expression of type II collagen and proteoglycan in ATDC5 cells cultured on various substrata; Polystryrene dish (PS), PDMAAm gel, P(AMPS-co-DMAAm)

(molar fraction of AMPS, F=0.5, 0.75), and PAMPS gel, in the absence of insulin. Scale bar, 100 μm. (A) Expression of type II collagen was analyzed by immunofluorescent staining at 14 days of culture. The cells were stained with an anti-type II collagen antibody (green) and Hoechst 33258 (red). (B) Expression of proteoglycan was analyzed by alcian blue staining at 14 days of culture.

Figure 5. Phase contrast microscopy images of ATDC5 cells cultured on various substrata; Polystryrene dish (PS), PDMAAm gel, P(AMPS-co-DMAAm) (molar fraction of AMPS, F=0.5), and PAMPS gel, in the presence of insulin at day 3 and 7. Scale bar, 100 μm.

Figure 6. Gene expression analysis of type I collagen (A), aggrecan (B), and type II collagen (C) in ATDC5 cells cultured on various substrata; Polystryrene dish (PS), PDMAAm gel, P(AMPS-co-DMAAm) (molar fraction of AMPS, F=0.5), and PAMPS gel, at 7 days after insulin treatment. Expression of each gene was measured by quantitative real-time PCR and normalized to GAPDH expression levels. Values are the mean ± S.D. obtained in four experiments. *$p < 0.05$, and ** $p < 0.01$versus polystyrene, by Dunnett's test.

Figure 7. Synthesis of type II collagen and proteoglycan in ATDC5 cells cultured on various substrata; Polystryrene dish (PS), PDMAAm gel, P(AMPS-co-DMAAm) (molar fraction of AMPS, F=0.5), and PAMPS gel, in the presence of insulin. Scale bar, 100 μm. (A) Expression of type II collagen was analyzed by immunofluorescent

staining at day 7. The cells were stained with an anti-type II collagen antibody (green) and Hoechst 33258 (red). (B) Expression of proteoglycan was analyzed by alcian blue staining at day 7.

Table 1: List of primers used in the real-time PCR analysis of gene expression in ATDC5 cells.

Primer ID	Primers (5′–3′)	Expected size (bp)	Accession No.
Collagen Type I–F	ATGCCGCGACCTCAAGATG	153	NM007742
Collagen Type I–R	TGAGGCACAGACGGCTGAGTA		
Collagen Type II–F	AGGGCAACAGCAGGTTCACATAC	171	NM031163
Collagen Type II–R	TGTCCACACCAAATTCCTGTTCA		
Aggrecan–F	AGTGGATCGGTCTGAATGACAGG	105	NM007424
Aggrecan–R	AGAAGTTGTCAGGCTGGTTTGGA		
GAPDH–F	TGTGTCCGTCGTGGATCTGA	150	NM001001303
GAPDH–R	TTGCTGTTGAAGTCGCAGGAG		

Figure 1.

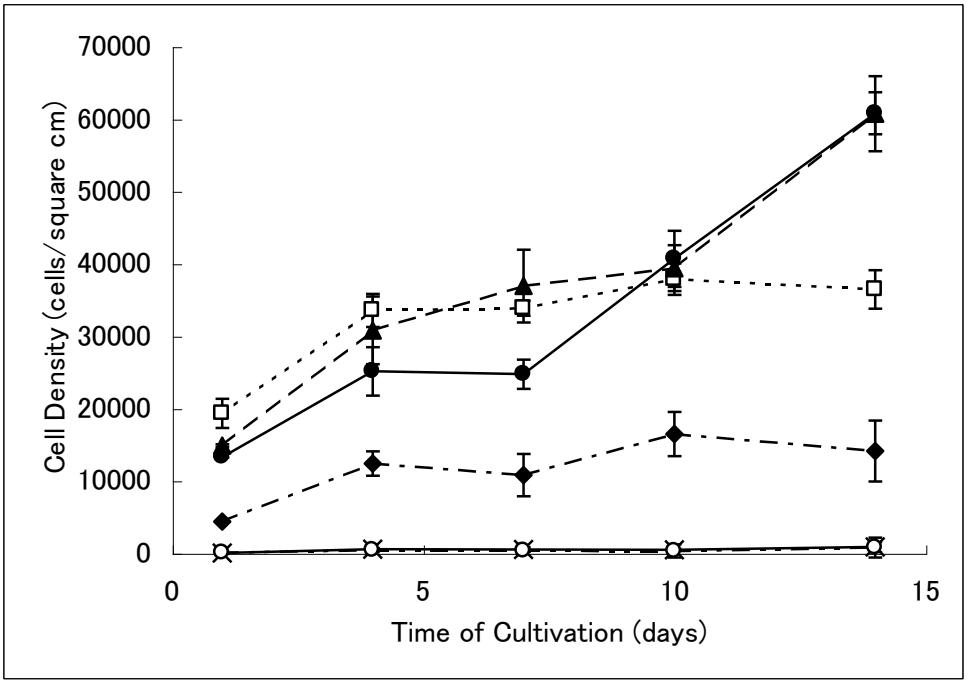
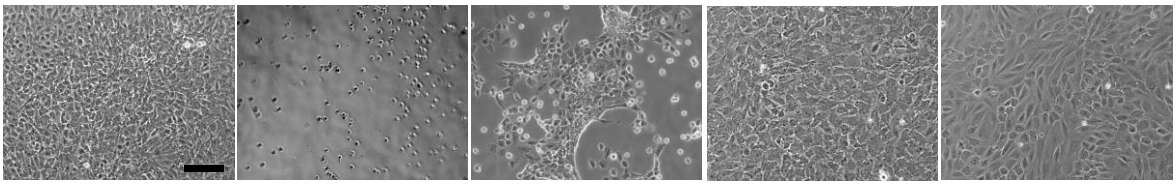

70000
60000
50000
40000
30000
20000
10000
0
Cell Density (cells/square cm)
0
5
10
15
Time of Cultivation (days)

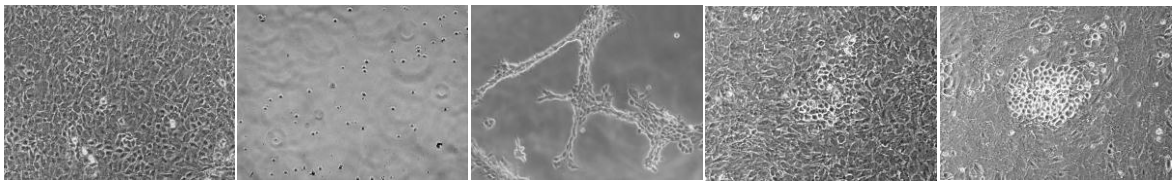
Figure 2.

Day 7



PS | PDMAAm | P(AMPS-co-DMAAm) (F=0.5) | P(AMPS-co-DMAAm) (F=0.75) | PAMPS

Day 14



PS | PDMAAm | P(AMPS-co-DMAAm) (F=0.5) | P(AMPS-co-DMAAm) (F=0.75) | PAMPS

Figure 3.

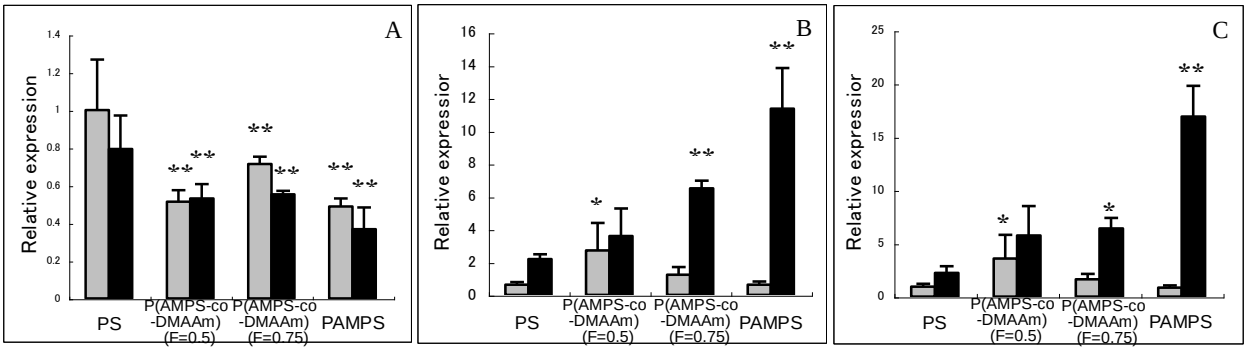

A
Relative expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PS
P(AMPS-co -DMAAm) (F=0.5)
P(AMPS-co -DMAAm) (F=0.75)
PAMPS
B
Relative expressior
16
14
12
10
8
6
4
2
0
C
Relative expressior
25
20
15
10
5
0

Figure 4.

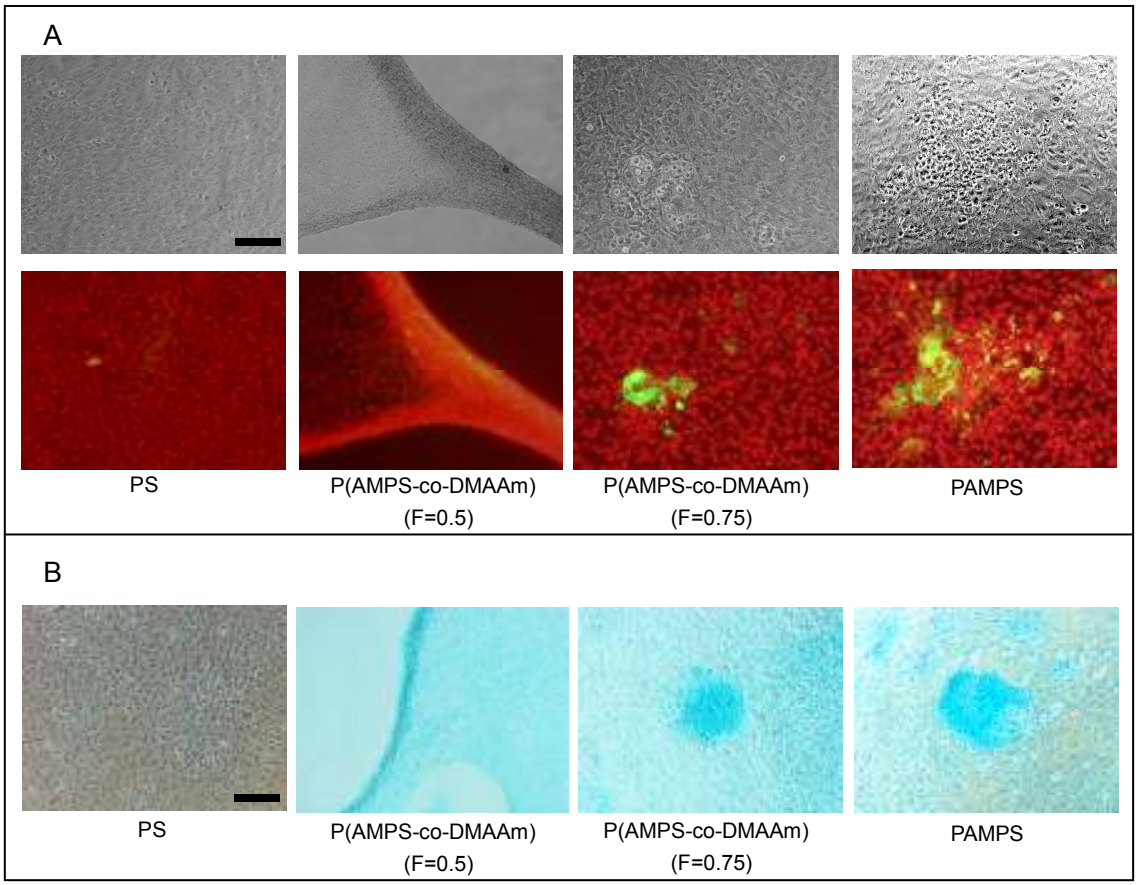

A
PS
P(AMPS-co-DMAAm)
(F=0.5)
P(AMPS-co-DMAAm)
(F=0.75)
PAMPS
B
PS
P(AMPS-co-DMAAm)
(F=0.5)
P(AMPS-co-DMAAm)
(F=0.75)
PAMPS

Figure 5.

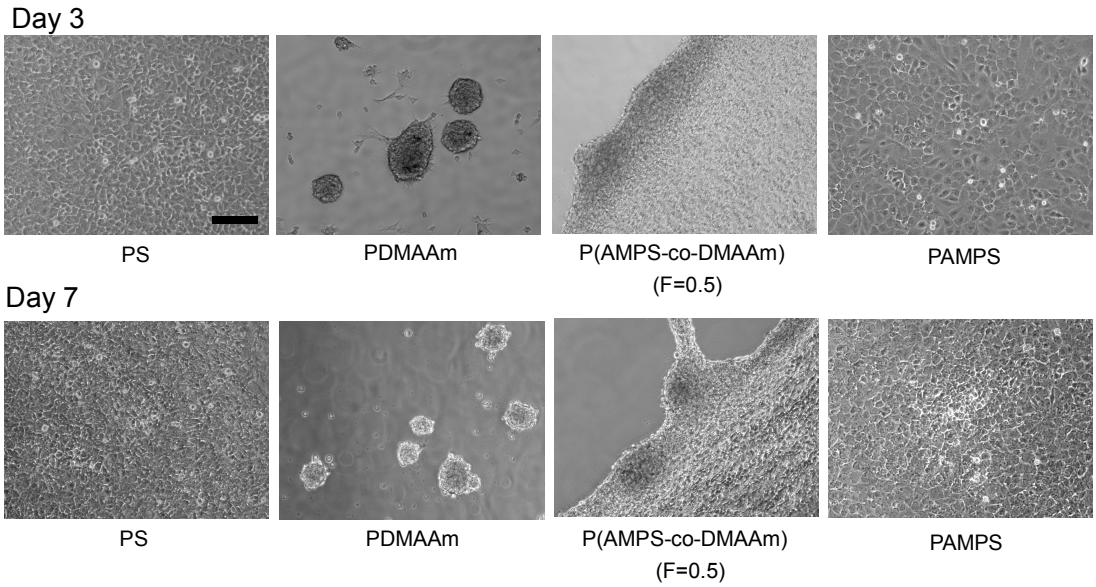

Day 3
PS
PDMAAm
P(AMPS-co-DMAAm)
(F=0.5)
PAMPS
Day 7
PS
PDMAAm
P(AMPS-co-DMAAm)
(F=0.5)
PAMPS

Figure 6.

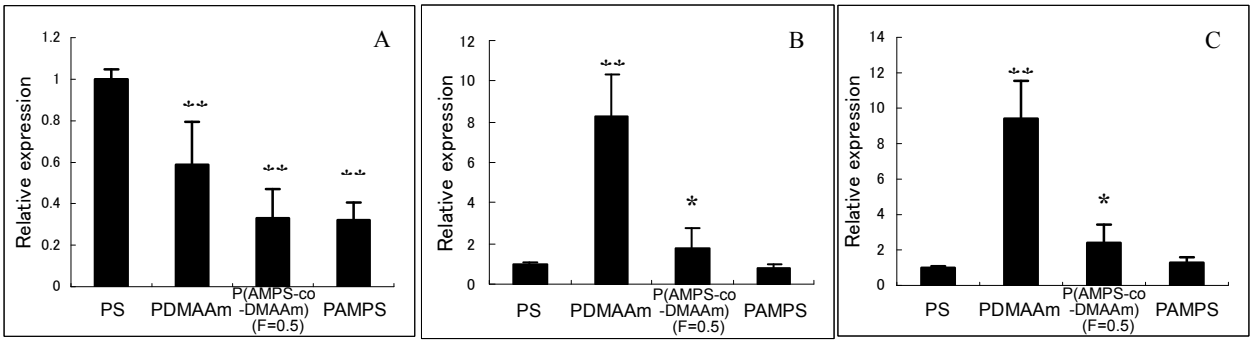
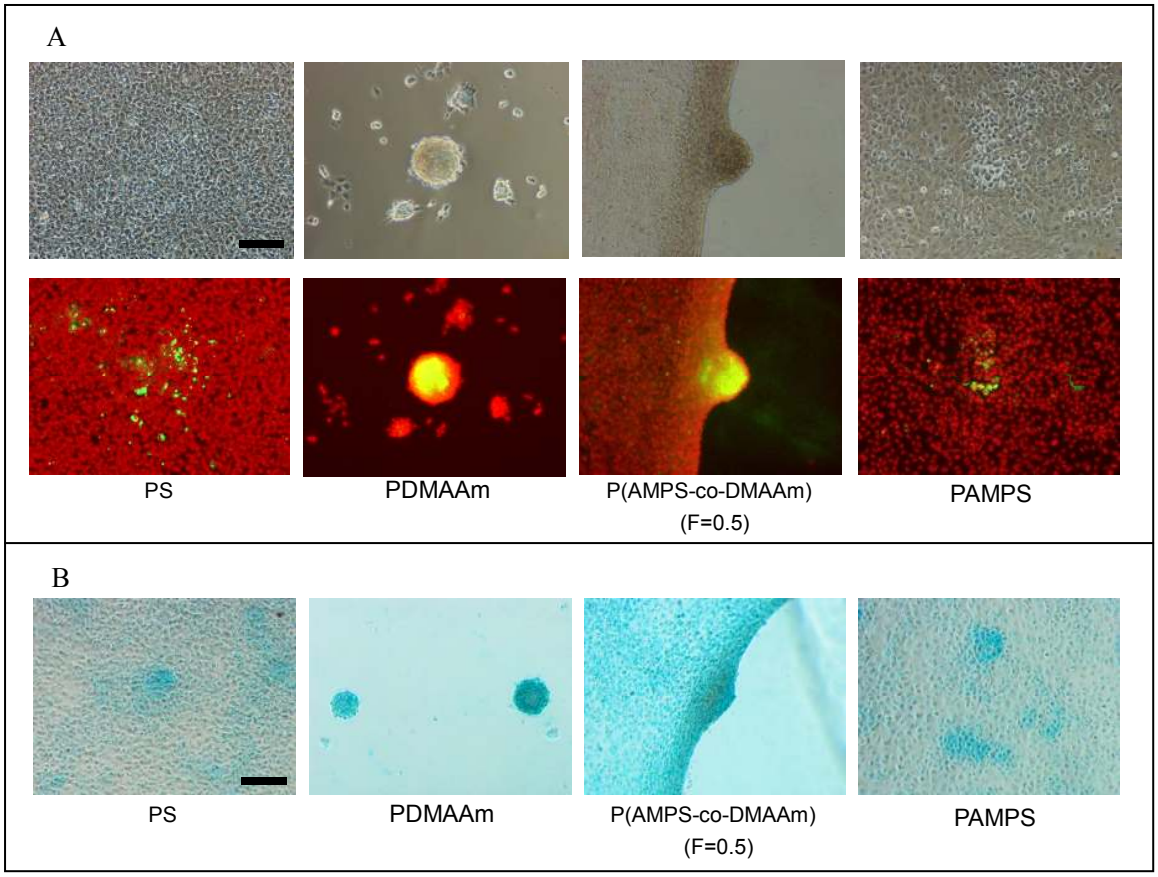

A
Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
**
**
**
PS
PDMAAm
P(AMPS-co -DMAAm) (F=0.5)
PAMPS
B
Relative expression
12
10
8
6
4
2
0
**
*
PS
PDMAAm
P(AMPS-co -DMAAm) (F=0.5)
PAMPS
C
Relative expression
14
12
10
8
6
4
2
0
**
*
PS
PDMAAm
P(AMPS-co -DMAAm) (F=0.5)
PAMPS

Figure 7.


A
PS
PDMAAm
P(AMPS-co-DMAAm)
(F=0.5)
PAMPS
B
PS
PDMAAm
P(AMPS-co-DMAAm)
(F=0.5)
PAMPS