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Immunological response to tissue-engineered cartilage derived from auricular chondrocytes and a PLLA scaffold in transgenic mice

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ABSTRACT

The immune response against biomaterials in tissue-engineered constructs could potentially worsen the outcome of tissue regeneration, but immunological reactions between host and donor in tissue-engineered constructs remain to be clarified. In the present study, we syngenically transplanted tissue-engineered cartilage constructs consisting of C57BL/6 mice auricular chondrocytes and poly-L-lactic acid scaffolds (MW:200,000) into EGFP transgenic mice of C57BL/6 background, and evaluated the response by the localization of donor-derived and host-derived cells, the latter of which were distinguished by the presence of EGFP. While donor-derived cells constituted the areas of regenerated cartilage, host-derived cells were increased in number for the initial two weeks, and then decreased and excluded to non-cartilage areas thereafter. Furthermore, EGFP positivity was mostly co-localized with that of F4/80, suggesting most of the host-derived cells in the tissue-engineered constructs could be macrophages. Immunohistochemical staining of the tissue-engineered cartilage constructs revealed expression of factors related to immune privilege in chondrocytes, such as macrophage migration inhibitory factor (MIF), fas ligand (FasL) and others. Co-culture of chondrocytes and macrophages in vitro increased the expression of MIF and FasL in the chondrocytes, suggesting that chondrocytes in tissue-engineered cartilage constructs could regulate the actions of host-derived macrophages by expressing factors related to immune privilege.

1. Introduction

Regenerative medicine using autologous cells is expected to be an alternative approach to replace allogenic organ transplantation, because of its reduced risk of immunogenic rejection. Transplantation of cells without any supportive material, however, would be hardly enough to reconstruct tissues with proper morphology and functions, so combined usage with a scaffold would be necessary. In cartilage, research on regenerative medicine is relatively advanced compared with that on other tissues, and some protocols have already been applied in clinical settings. Autologous chondrocytes have been implanted in patients with focal cartilage defects in joints since as early as the 1990s [1], while they have also been used for nasal augmentation by injection into subcutaneous pockets [2]. However, as these protocols utilize chondrocytes suspended in solution or gel, the regenerated cartilage might have some limitations in its biological and biophysical properties. To overcome such drawbacks in cell suspension, combined usage of biodegradable polymer scaffolds could be an effective option, since they can provide mechanical support efficiently.

Such rigid scaffolds to serve the purpose can be fabricated by employing biomaterials, such as biodegradable polymers. On the other hand, possible foreign body reaction to the scaffolds would promote the migration of immune cells and the secretion of catabolic factors at transplanted site, potentially hampering the process of tissue regeneration. Furthermore, immune response in tissue-engineered constructs might include autoimmune reaction against autologous cells besides the foreign body reaction against the scaffolds. Therefore, the tissue reactions in tissue-engineered constructs based on scaffolds could be extremely complex, remaining to be fully clarified.

Poly-L-lactic acid (PLLA) is a typical biodegradable polymer that has been safely used as intraosseous medical devices for decades [3]. Nonetheless, PLLA and its degradation products are not totally biological inert, and macrophages and multinucleated giant cells have been reported to appear on the surface of implanted PLLA screws in sheep bone [4]. In our previous study [5], we utilized PLLA porous scaffolds with human auricular chondrocytes to make

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tissue-engineered cartilage constructs, and transplanted the constructs subcutaneously into the back of athymic nude mice. Compared to the PLLA scaffolds transplanted alone, the tissue-engineered cartilage constructs showed a lower number of infiltrating macrophages and blood vessels with lower level of interleukin-1β. The exact molecular mechanism mediating such suppressed reaction in our previous study [5] remained to be determined with immunocompetent animals, as analysis of biological immune response can be limited in case of using athymic nude mice as host animals. Clarification on this issue in immunocompetent animals would be beneficial to modulate the tissue reactions, which might contribute to promote the maturation of tissue-engineered cartilage.

Immune privilege is known to be formed in certain tissues, where antigen normally would not elicit an immune reaction. Considering that cartilage is naturally devoid of blood vessels and is regarded to be immunologically privileged [6], there could be a possibility of immune privilege being involved in immunosuppressive actions in tissue-engineered cartilage. Our previous study using athymic nude mice suggested the possible involvement of macrophage migration inhibitory factor (MIF) in tissue-engineered cartilage [5], but several other factors related to immune privilege including MHC molecules [7], transforming growth factor-beta (TGF-β) [7], neuropeptides [8] and Fas ligand [9,10] have yet to be investigated. We hypothesized that donor-derived chondrocytes might form immune privilege by expressing these factors and modulate tissue reactions in the tissue-engineered cartilage. Since immune privilege was originally used to mean the phenomenon, in which transplanted grafts could survive without causing a T cell-mediated rejection reaction [10], the experimental model using athymic nude mice may not be sufficient for the evaluation of immune privilege.

To that end, in the present study, we established an animal experimental model of syngenic transplantation, in which tissue-engineered cartilage constructs consisting of C57BL/6 mice auricular chondrocytes and PLLA scaffolds were transplanted into the mice with the same genetic background. Because precise recognition of host-derived cells as well as donor-derived chondrocytes would be imperative to disclose the cytological mechanisms on the formation of the immune privilege, we used EGFP transgenic mice of C57BL/6 background as host animals, and examined the localization of host-derived and donor-derived cells by observing the difference in EGFP fluorescence. Furthermore, the expression of immune privilege factors was investigated to promote clear understanding of the formation of immune privilege in tissue-engineered cartilage.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12), penicillin–streptomycin solution, and trypsin–EDTA solution were purchased from Sigma Chemical Co. (MO, USA). Collagenase from Clostridium histolyticum was from Wako Pure Chemical Industries (Osaka, Japan), insulin was from MP Biomedicals (CA, USA), and FGF-2 was from Kaken Pharmaceutical Co, Ltd. (Tokyo, Japan). Other materials included 3% atelocollagen implant from Koken Co., Ltd. (Tokyo, Japan), PLLA porous scaffolds from KRI (Kyoto, Japan), anti-F4/80 antibody (Cl: A3-1) from BMA (Augst, Switzerland), anti-calcitonin gene-related peptide (CGRP) from BIOMOL International, L.P. (Exeter, UK), anti-α-melanocyte-stimulating hormone (α-MSH) antibody from BIOMOL International, L.P., anti-substance P antibody from GeneTex Inc. (TX, USA), anti-vasoactive intestinal peptide (VIP) antibody from Acris Antibodies GmbH (Herford, Germany), anti-macrophage migration inhibitory factor (MIF) antibody (FL-115) from Santa Cruz (CA, USA), anti-fas ligand (FasL) antibody from GeneTex Inc. (TX, USA), and anti-transforming growth factor-beta (TGF-β) antibody from Santa Cruz (CA, USA). The biotinylated secondary antibody, Vectastain Elite ABC Kit and Peroxidase Substrate kit DAB were all obtained from Vector Laboratories (CA, USA).

2.2. Cell culture

Human cartilage was obtained under informed consent from remnant auricular cartilage of microtia patients who underwent surgery at the University of Tokyo Hospital. All the procedures for the present experiments were approved by the ethics committee or institutional committee for animal research of the University of Tokyo Hospital (ethics permission #622), and the experiments was conducted according to the principles expressed in the declaration of Helsinki. Isolation and cell culture of human auricular cartilage was conducted as previously described [5]. Mice auricular chondrocytes were obtained by digesting the ear and external ear canal of 6-week-old C57BL/6 with 0.15% collagenase solution for 8 hrs, and cultured in DMEM/F12 supplemented with 5% FBS, 5 μg/ml insulin and 100 ng/ml FGF-2. Both human and mice auricular chondrocytes were cultured to passage 2. Mice macrophage-like cell line Mm1 was purchased from Riken Cell Bank (Tsukuba, Japan) and maintained in DMEM supplemented with 5% human serum. Co-culture of human auricular choncrocytes and Mm1 was carried out in 3D pellets according to previous studies [5,11]. We used a macrophage-like cell line to avoid possible cell contamination in primary macrophages, and examined the exact effects of macrophages in co-culture.

2.3. Fabrication of tissue-engineered cartilage and transplantation

PLLA scaffolds were produced by sugar-leaching method. The molecular weight of the scaffolds was 200,000, average pore size was 0.3 mm and average porosity was more than 95%. PLLA scaffolds of $4 \times 4 \times 3$ mm^3 were sterilized in 70% ethanol before use. Then, 1% atelocollagen gel, which was diluted from original 3% atelocollagen gel with DMEM/F12, was used as a cell suspension buffer to retain the chondrocytes in the scaffold efficiently. To make tissue-engineered constructs, mouse chondrocytes suspended in 1% atelocollagen gel (2×10^7 cells/200 μl) were applied to the PLLA scaffold and incubated at 37 °C in 5% CO_2 for 3 hrs (PLLA/Cell group, $n = 3$). At the same time, other scaffolds were allowed to absorb 200 μl DMEM/F12, followed by incubation as described above for the control group (PLLA group, $n = 3$). EGFP transgenic mice (C57BL/6-Tg(CAG-EGFP)C14-Y01-FM131Osb), which express EGFP under the CAG promoter throughout the body, were originally produced by Dr. M. Okabe (Osaka University, Osaka, Japan) and were provided by RIKEN BioResource Center (Tsukuba, Japan). Regarding the transplantation procedure, 6-week-old male EGFP transgenic mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). A small incision was made on the back in the midline, and two types of constructs (PLLA/Cell and PLLA) were transplanted subcutaneously in each animal. Since in our preliminary study, PLLA scaffolds transplanted alone into mice were not histologically different from those transplanted together with PLLA/Cell constructs on the other side of mouse back, the systemic effects of each construct on the other could be negligible. Two or 8 weeks after the operation, harvested constructs were assessed by histological and immunohistochemical analysis.

2.4. Histological and immunohistochemical staining

Each sample was fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 8-μm sections. The sections were stained with toluidine blue to detect proteoglycan, as well as with hematoxylin and eosin (H–E staining). To detect host-derived cells, sections were deparaffinized in xylene, hydrated with step-down concentrations of ethanol, immersed in PBS, and observed under a fluorescence microscope. The sections were also used for immunohistochemical staining for F4/80, VIP, α-MSH, substance P, CGRP, MIF, FasL and TGF-β according to previous studies and the manufacturers' instructions. Colocalization of EGFP with F4/80 or MIF was examined by immunofluorescent staining.

2.5. RNA isolation and realtime RT-PCR

Total RNA from cells co-cultured in a 3D environment for 7 days was isolated with Isogen and reverse-transcribed with PrimeScript reverse transcriptase and random hexamers. Gene expression was detected by realtime qPCR using the standard SYBR green method with an ABI 7500 Real-Time PCR System (Applied BioSystems, CA, USA). Standard templates were produced according to a previous study [11]. Sequences of primers were 5′-GCCCGGACAGGGTCTACA-3′ and 5′-CTTAGGCGAAGGTGGAGTTGTT-3′ for *MIF*; 5′-ATGAGCCAGACAAATGGAGG-3′ and 5′-AAGACAGTCCCCCTTGAGGT-3′ for *FASLG*; 5′-CACGTGGAGCTGTACCA-GAA-3′ and 5′-GAACCCGTTGATGTCCACTT-3′ for *TGFB1*; 5′-GCATGTGGTTTGGTTCCTCT-3′ and 5′-GCACATTCAGAAGCAGGACA-3′ for CGRP; 5′-GAAGGTGAAGGTCGGAGTCA-3′ and 5′-GAAGATGGTGATGGGATTTC-3′ for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). All the primers have been confirmed to be specific to only the human gene. Transcript levels were normalized to that of *GAPDH*.

2.6. Statistics

Data are expressed as mean ± SD. Statistical significance was evaluated using Bonferroni test for multiple comparisons. A value of $p < 0.05$ was interpreted to denote statistical significance.

3. Results

3.1. Cartilage regeneration in tissue-engineered cartilage constructs

We made tissue-engineered constructs using a PLLA porous scaffold with or without C57BL/6 mouse auricular chondrocytes (PLLA/Cell group and PLLA group), and transplanted them subcutaneously into the back of C57BL/6-Tg(CAG-EGFP)C14-Y01-FM131Osb mice, EGFP transgenic mice with a syngenic C57BL/6 background. Both at 2 and 8 weeks after transplantation, toluidine blue staining of the PLLA scaffold only (PLLA group) did not show any distinctive metachromasia, an indicator of proteoglycan accumulation (Fig. 1; TB). No cartilage regeneration was observed in H–E-stained PLLA constructs, and cells with a low C/N ratio, which were suggested to be hematopoietic cells, were accumulated around the PLLA (Fig. 1; HE). Fluorescent observation of sections, which were also examined in a bright field, revealed intense infiltration of host-derived cells in the PLLA group even at 2 weeks (Fig. 1; BF and EGFP).

In contrast, toluidine blue staining of the tissue-engineered constructs in the PLLA/Cell group demonstrated an increase in areas of metachromasia with time, and mature cartilage was observed throughout the engineered tissue by 8 weeks, which was maintained at 6 months after transplantation (Fig. 2). H–E staining

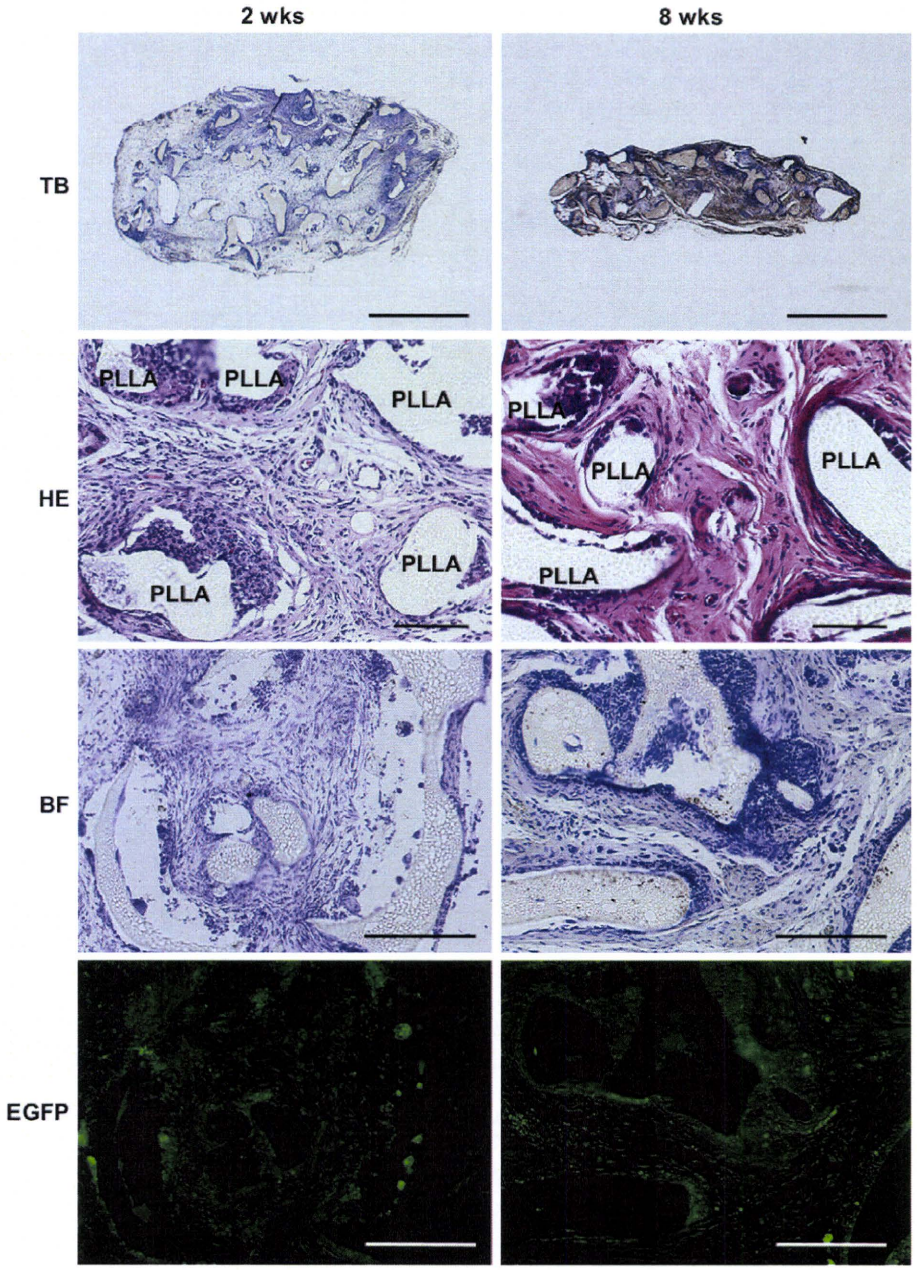

Fig. 1. Histological findings of PLLA scaffolds without cells (PLLA group), transplanted subcutaneously on the back of EGFP transgenic mice. (**TB**) Toluidine blue staining. Scale bars, 1 mm. (**HE**) Hematoxylin and eosin (H–E) staining. Scale bars, 100 μm. (**BF and EGFP**) Observation of sections under EGFP fluorescence and corresponding bright field (BF) revealed intense infiltration of host-derived cells in PLLA constructs at 2 weeks. Scale bars, 100 μm.

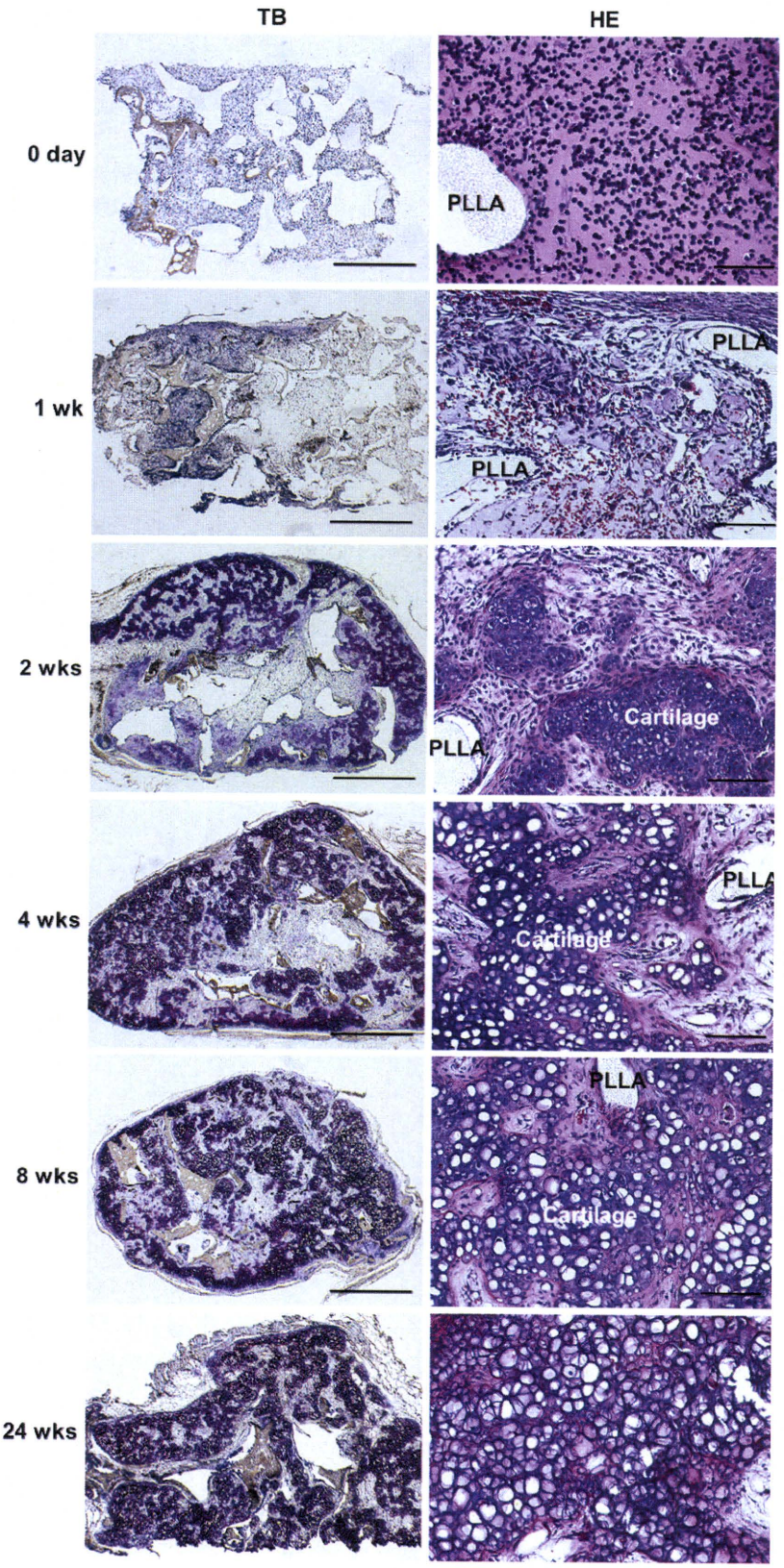

Fig. 2. Histological findings in toluidine blue staining (**TB**) and H–E staining (**HE**) of tissue-engineered constructs consisting of mouse auricular chondrocytes and PLLA scaffolds (PLLA/Cell group) up to 24 weeks after transplantation. The areas of metachromasia increased with time, and mature cartilage was observed throughout the tissue-engineered constructs by 8 weeks, which was maintained throughout the observation period. Scale bars, 1 mm for TB and 100 μm for HE.

demonstrated invasion of hematocytes into the PLLA/Cell constructs at 1 week. Subsequently, the cells in the tissue-engineered constructs acquired a chondrocyte-like appearance, and formation of extracellular matrix became noticeable at 2 weeks. By 8 weeks, most of cells showed specific features of differentiated chondrocytes, representing cartilage regeneration. Regenerated cartilage was sustained without any obvious histological changes at 6 months (Fig. 2).

3.2. Origins of cells in engineered constructs

To identify the origins of the cells in the engineered constructs as host-derived or donor-derived, we distinguished host-derived cells from donor-derived cells by their green fluorescence. As early as 1 week after transplantation, the host-derived cells in the PLLA/Cell group were evenly scattered throughout the engineered constructs, and were mingled with the donor-derived cells (Fig. 3; 1 week). Subsequently, the distribution of the host-derived cells became uneven at 2 weeks. The donor-derived cells formed small clusters, among which host-derived cells were located unevenly (Fig. 3; 2 weeks). By 8 weeks, the green-fluorescence-positive host-derived cells were greatly reduced, and were restricted to the non-cartilage area, while the areas of cartilage consisted of chondrocytes derived from the donor (Fig. 3; 8 weeks). On the other hand, the constructs without cells (PLLA group) were more susceptible to invasion of host-derived cells than constructs in the PLLA/Cell group, and the number of host-derived cells in the PLLA group did not decrease with time as seen in the PLLA/Cell group

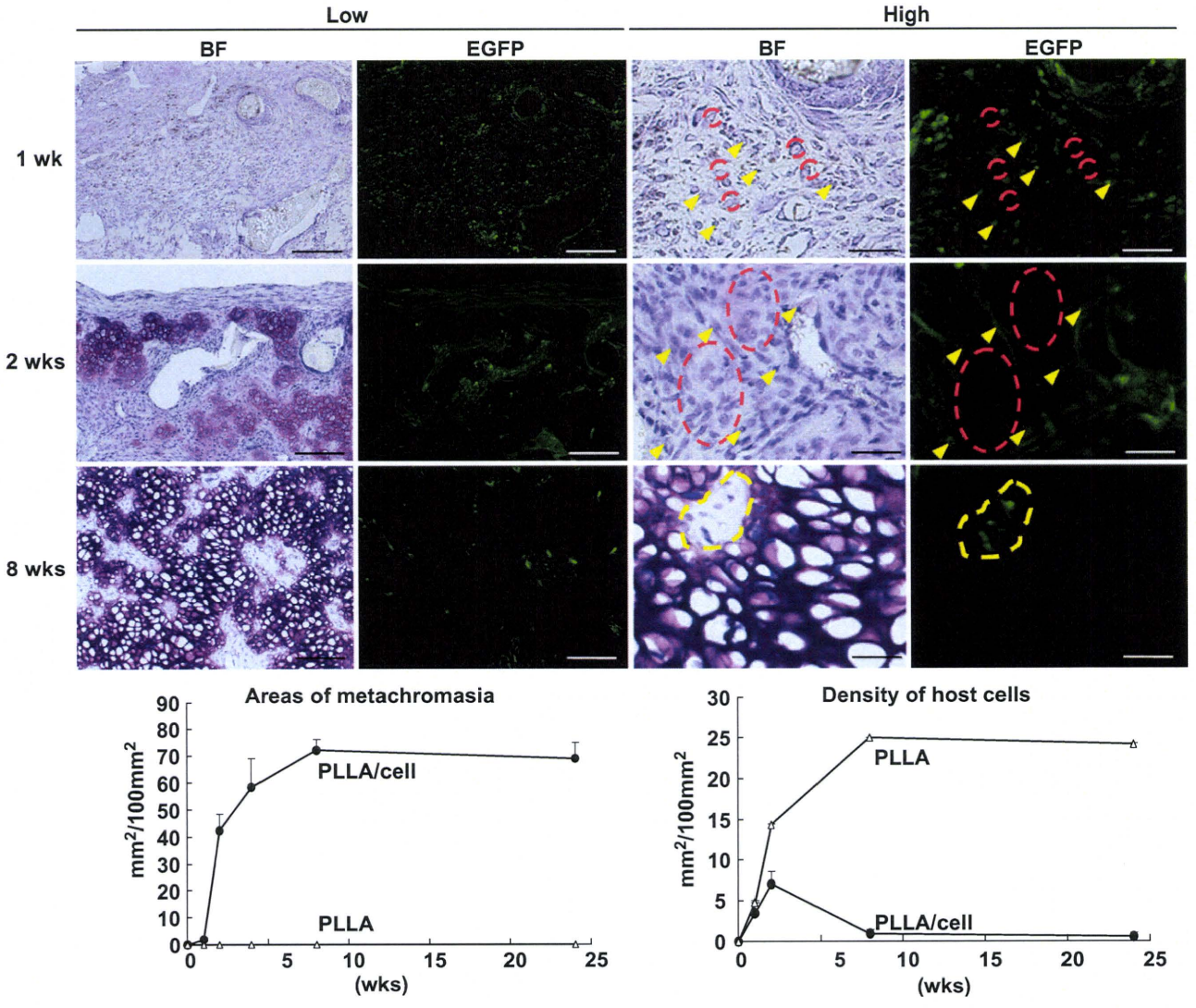

Fig. 3. Visualization of host-derived cells by EGFP fluorescence in tissue-engineered constructs consisting of mouse auricular chondrocytes and PLLA scaffolds (PLLA/Cell group). (1 week) Host-derived cells were evenly scattered throughout the engineered constructs (arrow heads), and mingled with donor-derived cells (dotted circles) at 1 week after transplantation. (2 weeks) Donor-derived cells formed small clusters (dotted circles), among which the host-derived cells were located in a spotted pattern (arrow heads) at 2 weeks. (8 weeks) Host-derived cells positive for green-fluorescence were restricted to non-cartilage areas, while the areas of cartilage consisted of chondrocytes derived from the donor by 8 weeks. **Low**, lower magnification. **High**, higher magnification. Scale bars, 100 μm for Low and 400 μm for High. (**Areas of metachromasia**) Areas of metachromasia on toluidine blue staining in the PLLA/Cell constructs gradually increased with time and reached a plateau at about 75% of the total engineered construct area by 8 weeks, while PLLA scaffolds did not show any distinctive metachromasia (**Density of host cells**) The density of host-derived cells in the PLLA/Cell constructs reached a peak around 2 weeks after transplantation. On the other hand, constructs in the PLLA group were more susceptible to invasion of host-derived cells, which did not decrease with time. Data are expressed as mean (bars) ± s.d. (error bars).

(Fig. 1 and Fig. 3; Density of host cells). Histomorphometric analysis of the engineered constructs in the PLLA/Cell group demonstrated that the areas of host-derived cells increased sharply by 2 weeks and gradually diminished thereafter (Fig. 3; Density of host cells), while the areas of metachromasia on toluidine blue staining gradually increased with time and reached a plateau at about 75% of the total engineered construct area by 8 weeks, which was maintained during the observation period for 24 weeks (Fig. 3; Areas of metachromasia).

3.3. Characteristics of host-derived cells in engineered constructs

To examine the cell characteristics of the host-derived cells constituting the engineered cartilage constructs, we conducted immunohistochemical staining for F4/80 antigen. Cells positive for the macrophage marker F4/80 were localized in the PLLA/Cell constructs at 2 weeks (Fig. 4; F4/80), while EGFP positivity, which indicated the host-derived cells in the present study, was mostly co-localized with that of F4/80 (Fig. 4; EGFP, Merged), suggesting that most of the host-derived cells in the PLLA/Cell constructs could be macrophages. Immunohistochemical staining for CD3 antigen for T lymphocytes, CD45 antigen for B lymphocytes, and NIMP-R14 for neutrophils was hardly observed in any group (data not shown).

3.4. Expression of factors related to immune privilege

Our previous and the present studies suggested that tissue reactions in tissue-engineered constructs based on a PLLA scaffold could be suppressed with the maturation of tissue-engineered cartilage (Fig. 3). Since cartilage is originally free from blood vessels and has been reported to be a site with immune privilege, we postulated that immune privilege factors could be involved in the suppression of tissue reactions, in which macrophages were mainly involved, in the tissue-engineered cartilage. We therefore investigated the expression of several factors related to immune privilege, including vasoactive intestinal peptide (VIP), α-melanocyte-stimulating hormone (α-MSH), substance P, calcitonin gene-related peptide (CGRP), macrophage migration inhibitory factor (MIF), fas ligand (FasL) and transforming growth factor-beta (TGF-β). Immunohistochemical analysis of these factors in tissue-engineered constructs demonstrated distinct expression of CGRP, MIF, FasL and TGF-β in the regenerated cartilage at 2 weeks after transplantation (Fig. 5; CGRP, MIF, FasL, TGF-β). Positivity for other factors, all of which were neuropeptides, was not so apparent (Fig. 5; VIP, α-MSH, substance P).

3.5. Co-culture of chondrocytes and Mm1

Since the chondrocytes in the tissue-engineered cartilage showed expression of some factors related to immune privilege, we next determined if the chondrocytes expressed these factors naturally or through interactions with the host-derived cells. Therefore, we conducted co-culture of human auricular chondrocytes and a mouse macrophage cell line, Mm1, at various ratios in chondrogenic medium, and investigated the expression of the factors in the chondrocytes by realtime RT-PCR. Among the factors investigated, the expression of *MIF*, which was detectable even in chondrocytes cultured alone, increased two-fold when co-cultured with Mm1 at a ratio of 19:1 (Fig. 5; MIF expression). The expression profile of *FASL* in the chondrocytes was similar, though the increase of expression in the co-culture was rather sharp compared to that of *MIF* (Fig. 5; FasL expression). On the other hand, the expression of *TGF-β* and *CGRP* in chondrocytes was unchanged regardless of the existence of co-cultured macrophages (Fig. 5 TGF-β and CGRP expression). Since the primers we used in the present study were designed to be specific to humans and not to mice, no expression was detectable in the group containing Mm1 only. In fact, double staining for MIF antigen and EGFP verified the expression of MIF in the donor-derived chondrocytes of the PLLA/Cell constructs, supporting that the factor secreted by the chondrocytes participated in the development of immune privilege in tissue-engineered cartilage (Fig. 6).

4. Discussion

The PLLA scaffold without cells (PLLA group), which served as control in the present study, underwent abundant infiltration of multinucleated giant cells and macrophages with subsequent formation of fibrous tissue, demonstrating the typical features of foreign body reaction. On the other hand, the tissue-engineered constructs containing chondrocytes (PLLA/Cell group) also shared these histological features, but to a lesser extent. Since host-derived cells in the present study were shown to be mainly macrophages and not T cells, the tissue reactions in the tissue-engineered cartilage constructs may be caused by the PLLA scaffold as a foreign body rather than by a rejection reaction to syngenically transplanted chondrocytes.

Foreign body reactions are characterized by three phases: onset, progression and resolution [12]. In the case of tissue-engineered constructs, macrophages could be problematic during the progression phase in some ways. First of all, soluble factors produced by macrophages, such as IL-1, IL-6, IL-10, IL-12, IL-18, TNF-α, TGF-β, IL-8, MCP-1 and others [12,13], would affect the viability of donor-derived cells in the constructs. In cartilage regeneration, a catabolic cytokine IL-1β has the potential to prompt chondrocytes to secrete aggrecanase and MMPs [14,15], which could cause deterioration of the accumulation of proteoglycan in the engineered cartilage. Secondly, macrophages influence the actions of lymphocytes [16], which might also enhance the intensity of tissue reactions, resulting in the impairment of regenerated

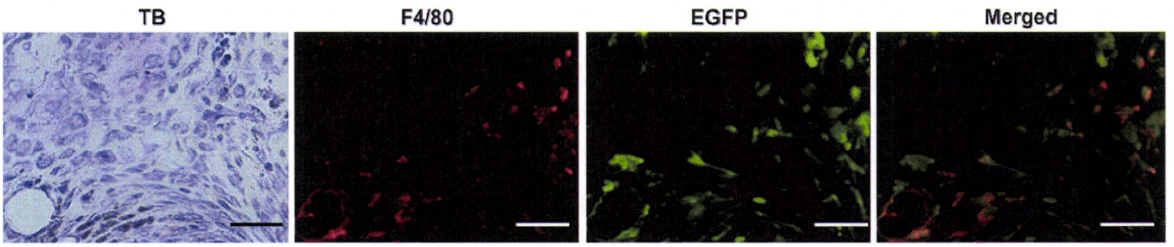

Fig. 4. Localization of macrophages and host-derived cells in tissue-engineered constructs consisting of mouse auricular chondrocytes and PLLA scaffolds (PLLA/Cell group) at 2 weeks after transplantation. Double-immunostaining for F4/80 and EGFP showed that most EGFP positivity was co-localized with that of F4/80. **TB**, toluidine blue staining: **F4/80**, immunohistochemical staining for F4/80 antigen: **EGFP**, immunohistochemical staining for EGFP antigen: **Merged**, merged image of EGFP and F4/80. Scale bars, 100 μm.

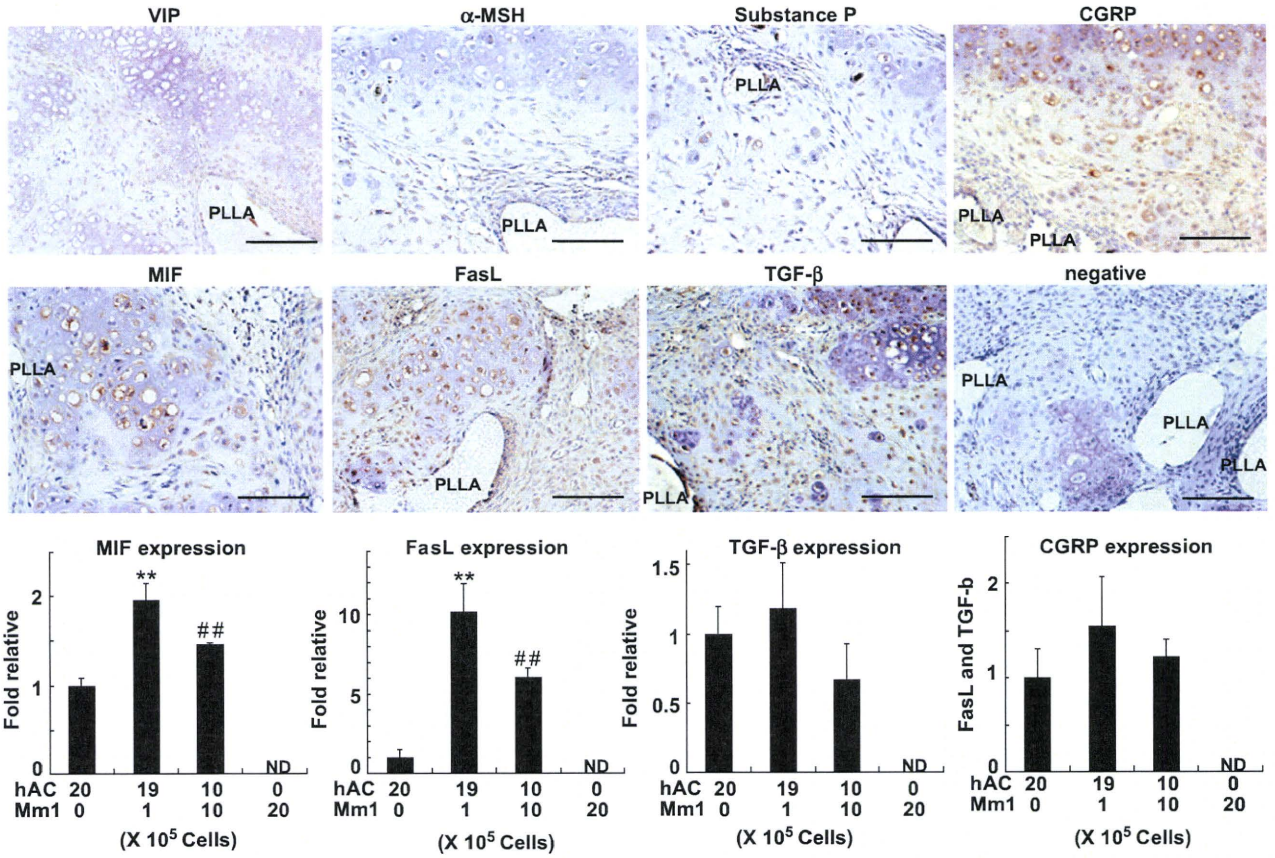

Fig. 5. Expression of immune privilege factors in tissue-engineered constructs consisting of mouse auricular chondrocytes and PLLA scaffolds (PLLA/Cell group) and in co-culture of human chondrocytes and mouse macrophages in vitro. **Top**, Immunostaining for vasoactive intestinal peptide (VIP), α-melanocyte-stimulating hormone (α-MSH), substance P, calcitonin gene-related peptide (CGRP), macrophage migration inhibitory factor (MIF), fas ligand (FasL), and transforming growth factor-beta (TGF-β) revealed positivity of CGRP, MIF, FasL and TGF-β in regenerated cartilage of PLLA/Cell constructs at 2 weeks after transplantation. Scale bars, 100 μm. **Bottom**, Expression of immune privilege factors in human auricular chondrocytes co-cultured with mouse macrophage cell line, Mm1, with various ratios in chondrogenic medium for 1 week. Data are expressed as mean (bars) ± s.d. (error bars). **, $p < 0.01$, vs. groups with ratio of 20:0 and 10:10. ##, $p < 0.01$, vs. group with ratio of 20:0.

tissues. In our previous study transplanting human tissue-engineered cartilage constructs subcutaneously into the back of athymic nude mice [5], the regenerated cartilage showed less infiltration of host-derived inflammatory cells in comparison with that in the present study. Considering the restricted immune functions in athymic mice, which are almost completely devoid of T cells, the difference in the intensity of host reactions between our two studies may be attributable to the functions of T cells, although T cells could hardly be found in and around areas of tissue-engineered constructs in the present study. As macrophages could sensitize T cells to proliferate, it is likely that macrophages would impair the regeneration of tissue-engineered constructs through cross-talk with T cells.

While playing pivotal roles in the maintenance of inflammatory processes during the progression phase of foreign body reactions, macrophages in the resolution phase are known to provoke fibrosis and capsulation by secreting TGF-β [17]. Given that TGF-β is a potent inducer of ECM formation and chondrocyte differentiation [18,19], maturation of tissue-engineered cartilage could be facilitated by residual macrophages once the reactions reach the resolution phase. Indeed, the density of host-derived cells in the present study showed a sharp peak at 2 weeks, while maturation of cartilage proceeded markedly after that, suggesting a possible transition between progression and resolution at around 2–3 weeks after transplantation. If the resolution phase proceeds favorably for maturation of tissue-engineered cartilage constructs, a requirement for

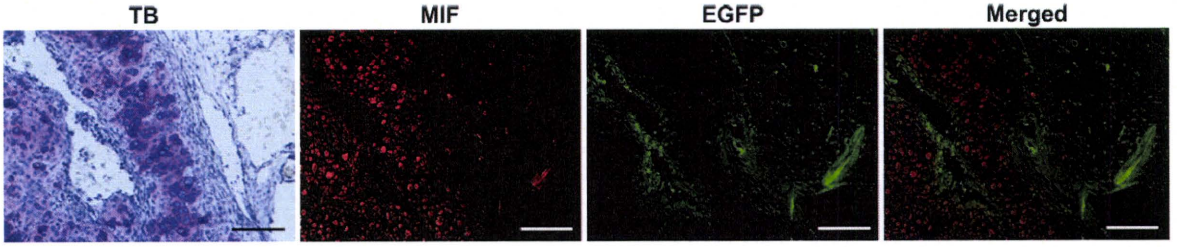

Fig. 6. Immunolocalization of MIF in tissue-engineered constructs consisting of mice auricular chondrocytes and PLLA scaffolds (PLLA/Cell group) at 2 weeks after transplantation. Double-immunostaining for MIF and EGFP showed that MIF was localized in the areas negative for EGFP, host-derived cells. **TB**, toluidine blue staining: **MIF**, immunohistochemical staining for MIF antigen: **EGFP**, immunohistochemical staining for EGFP antigen: **Merged**, merged image of EGFP and MIF. Scale bars, 100 μm.

successful cartilage regeneration would be to maintain the viability of donor-derived cells during the progression phase, presumably for the initial several weeks.

Based on our present findings, host-derived cells in the tissue-engineered constructs were excluded to non-cartilage areas with time, while chondrocytes expressed some factors related to immune privilege, such as MIF, FasL, TGF-β and CGRP [7–9,20]. Moreover, expression of *MIF* and *FasL* in chondrocytes was shown to increase under co-culture with Mm1, suggesting that the chondrocytes could enhance the expression of these factors in response to the host-derived macrophages. Therefore, our results indicated a possibility that cytokines or humoral factors secreted by macrophages during the foreign body reaction against scaffold-based regenerated tissues might induce chondrocytes to form immune privilege, in which chondrocytes actively regulated the localization and activity of macrophages.

MIF was originally found as a factor that inhibited the migration of macrophages [21], and subsequent studies have reported other biological functions including immune privilege [20,22–25]. Although its specific function in tissue-engineered cartilage constructs remains to be clarified, MIF may regulate the migration and localization of macrophages, and modulate the intensity of tissue reactions. Regarding the functions of FasL, one study demonstrated that genetically transferred FasL in chondrocytes could enhance apoptosis of activated T cells and be beneficial for allogenic transplantation of tissue-engineered cartilage [26]. Although the infiltrated immune cells in our tissue-engineered cartilage were mainly macrophages and not T cells, which was probably because our study utilized syngenic transplantation containing a biodegradable scaffold, the mechanism of FasL inducing apoptosis in Fas-presenting immune cells [27] might explain the suppressed tissue reactions in the present study as well. On the other hand, local or partial inhibition of immune responses could potentially impair the complicated network of immune reactions. Indeed, another study reported that inoculated tumor cells expressing FasL on their surface induced processing and secretion of IL-1β in peritoneal exudate cells in mice, suggesting that ectopically over-expressed FasL could be inflammatory [28]. As the biological functions of these immune privilege factors are multiple and enigmatic, further investigations would be necessary to specify their functions in tissue-engineered cartilage constructs.

5. Conclusions

The present study clarified the localization of host-derived and donor-derived cells in transplanted tissue-engineered cartilage over 6 months by using EGFP transgenic mice, and demonstrated that the action of host-derived macrophages could be modulated by immune privilege factors expressed by donor-derived chondrocytes.

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Appendix

Figure with essential color discrimination. All of the figures in this article have parts that are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.10.053.

References

[1] Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 1994;331(14):889–95.
[2] Yanaga H, Yanaga K, Imai K, Koga M, Soejima C, Ohmori K. Clinical application of cultured autologous human auricular chondrocytes with autologous serum for craniofacial or nasal augmentation and repair. Plast Reconstr Surg 2006;117(6):2019–30. discussion 31–2.
[3] Kulkarni RK, Pani KC, Neuman C, Leonard F. Polylactic acid for surgical implants. Arch Surg 1966;93(5):839–43.
[4] Walton M, Cotton NJ. Long-term in vivo degradation of poly-L-lactide (PLLA) in bone. J Biomater Appl 2007;21(4):395–411.
[5] Fujihara Y, Asawa Y, Takato T, Hoshi K. Tissue reactions to engineered cartilage based on poly-L-lactic acid scaffolds. Tissue Eng Part A 2009;15(7):1565–77.
[6] Bolano L, Kopta JA. The immunology of bone and cartilage transplantation. Orthopedics 1991;14(9):987–96.
[7] Wilbanks GA, Streilein JW. Fluids from immune privileged sites endow macrophages with the capacity to induce antigen-specific immune deviation via a mechanism involving transforming growth factor-beta. Eur J Immunol 1992;22(4):1031–6.
[8] Ferguson TA, Fletcher S, Herndon J, Griffith TS. Neuropeptides modulate immune deviation induced via the anterior chamber of the eye. J Immunol 1995;155(4):1746–56.
[9] Bellgrau D, Gold D, Selawry H, Moore J, Franzusoff A, Duke RC. A role for CD95 ligand in preventing graft rejection. Nature 1995;377(6550):630–2.
[10] Barker CF, Billingham RE. Immunologically privileged sites. Adv Immunol 1977;25:1–54.
[11] Liu G, Kawaguchi H, Ogasawara T, Asawa Y, Kishimoto J, Takahashi T, et al. Optimal combination of soluble factors for tissue engineering of permanent cartilage from cultured human chondrocytes. J Biol Chem 2007;282(28):20407–15.
[12] Luttikhuizen DT, Harmsen MC, Van Luyn MJ. Cellular and molecular dynamics in the foreign body reaction. Tissue Eng 2006;12(7):1955–70.
[13] Anderson JM, Rodriguez A, Chang DT. Foreign body reaction to biomaterials. Semin Immunol 2008;20(2):86–100.
[14] Beekman B, Verzijl N, de Roos JA, TeKoppele JM. Matrix degradation by chondrocytes cultured in alginate: IL-1 beta induces proteoglycan degradation and proMMP synthesis but does not result in collagen degradation. Osteoarthr Cartil 1998;6(5):330–40.
[15] Loeser RF. Molecular mechanisms of cartilage destruction: mechanics, inflammatory mediators, and aging collide. Arthritis Rheum 2006;54(5):1357–60.
[16] Brodbeck WG, Macewan M, Colton E, Meyerson H, Anderson JM. Lymphocytes and the foreign body response: lymphocyte enhancement of macrophage adhesion and fusion. J Biomed Mater Res A 2005;74(2):222–9.
[17] Ashcroft GS. Bidirectional regulation of macrophage function by TGF-beta. Microbes Infect 1999;1(15):1275–82.
[18] Moulharat N, Lesur C, Thomas M, Rolland-Valognes G, Pastoureau P, Anract P, et al. Effects of transforming growth factor-beta on aggrecanase production and proteoglycan degradation by human chondrocytes in vitro. Osteoarthr Cartil 2004;12(4):296–305.
[19] Moses HL, Serra R. Regulation of differentiation by TGF-beta. Curr Opin Genet Dev 1996;6(5):581–6.
[20] Apte RS, Sinha D, Mayhew E, Wistow GJ, Niederkorn JY. Cutting edge: role of macrophage migration inhibitory factor in inhibiting NK cell activity and preserving immune privilege. J Immunol 1998;160(12):5693–6.
[21] Bloom BR, Bennett B. Mechanism of a reaction in vitro associated with delayed-type hypersensitivity. Science 1966;153(731):80–2.
[22] Hagemann T, Robinson SC, Thompson RG, Charles K, Kulbe H, Balkwill FR. Ovarian cancer cell-derived migration inhibitory factor enhances tumor growth, progression, and angiogenesis. Mol Cancer Ther 2007;6(7):1993–2002.
[23] Roger T, David J, Glauser MP, Calandra T. MIF regulates innate immune responses through modulation of toll-like receptor 4. Nature 2001;414(6866):920–4.
[24] Mitchell RA, Liao H, Chesney J, Fingerle-Rowson G, Baugh J, David J, et al. Macrophage migration inhibitory factor (MIF) sustains macrophage proinflammatory function by inhibiting p53: regulatory role in the innate immune response. Proc Natl Acad Sci U S A 2002;99(1):345–50.
[25] Zhou Q, Yan X, Gershan J, Orentas RJ, Johnson BD. Expression of macrophage migration inhibitory factor by neuroblastoma leads to the inhibition of antitumor T cell reactivity in vivo. J Immunol 2008;181(3):1877–86.
[26] Xie GH, Wang SJ, Wang Y, Zhang Y, Zhang HZ, Jin S, et al. Fas ligand gene transfer enhances the survival of tissue-engineered chondrocyte allografts in mini-pigs. Transpl Immunol 2008;19(2):145–51.
[27] Chavez-Galan L, Arenas-Del Angel MC, Zenteno E, Chavez R, Lascurain R. Cell death mechanisms induced by cytotoxic lymphocytes. Cell Mol Immunol 2009;6(1):15–25.
[28] Miwa K, Asano M, Horai R, Iwakura Y, Nagata S, Suda T. Caspase 1-independent IL-1beta release and inflammation induced by the apoptosis inducer Fas ligand. Nat Med 1998;4(11):1287–92.

Selection of highly osteogenic and chondrogenic cells from bone marrow stromal cells in biocompatible polymer-coated plates

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Abstract: To enrich the subpopulation that preserves self-renewal and multipotentiality from conventionally prepared bone marrow stromal cells (MSCs), we attempted to use 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer-coated plates that selected the MSCs with strong adhesion ability and evaluated the proliferation ability or osteogenic/chondrogenic potential of the MPC polymer-selected MSCs. The number of MSCs that were attached to the MPC polymer-coated plates decreased with an increase in the density of MPC unit (0–10%), whereas no significant difference in the proliferation ability was seen among these cells. The surface epitopes of CD29, CD44, CD105, and CD166, and not CD34 or CD45, were detectable in the cells of all MPC polymer-coated plates, implying that they belong to the MSC category. In the osteogenic and chondrogenic induction, the MSCs selected by the 2–5% MPC unit composition showed higher expression levels of osteoblastic and chondrocytic markers (COL1A1/ALP, or COL2A1/COL10A1/Sox9) at passage 2, compared with those of 0–1% or even 10% MPC unit composition, while the enhanced effects continued by passage 5. The selection based on the adequate cell adhesiveness by the MPC polymer-coated plates could improve the osteogenic and chondrogenic potential of MSCs, which would provide cell sources that can be used to treat the more severe and various bone/cartilage diseases. © 2009 Wiley Periodicals, Inc. J Biomed Mater Res 92A: 1273–1282, 2010

Key words: bone marrow stromal cell (MSC); 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer; osteogenesis; chondrogenesis; cell adhesion

INTRODUCTION

Bone marrow mesenchymal stem cells or stromal cells (MSCs) retain the potential to differentiate into multiple cell lineages that include osteoblasts, chondrocytes, adipocytes, myoblasts, and early progenitors of neural cells.[1–3] Because MSCs can be easily obtained from a small aspirate of bone marrow and they rapidly proliferate during the early passages of the expansion culture, human MSCs are regarded as one of the attractive cell sources for regenerative medicine in bone, cartilage, heart, nerve, and other tissues. However, MSCs are principally collected from bone marrow aspirates only through their selection by adhesiveness onto the plastic culture dishes,[4] and therefore, they include various subpopulations of cells which possess different proliferation rates or differentiation potentials. During the long-term culture with repeated passages, the balance among the subpopulations in the MSCs changes as a result of the difference in the proliferation rates, which may cause a deterioration of the self-renewal property or multipotentiality after repeated passages.[5]

To isolate or enrich the subpopulation that preserves the self-renewal and the multipotentiality

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from the conventionally prepared MSCs, various kinds of efforts have been made in the past decade. It was reported that the sizes and structures of the cells could distinguish the cells possessing a great potential for multilineage differentiation, termed rapid self-renewal (RS) cells, from the heterogeneity of the MSCs.[6] The RS cells had a shaped round shape with approximately a 7-μm diameter, and could be purified by using a 10-μm filter.[6] However, some limitations had been pointed out in the paper that the filtration process could only provide a low yield of purified RS cells because the other-sized cells rapidly obstructed the filter pores. The RS cells were also characterized by the low forward scatter and low side scatter of light during a flow cytometric analysis.[7] During cell sorting with the criteria of a low forward scatter and low side scatter, the subpopulation was successfully enriched for the RS cells, which increased the differentiation potential for osteoblasts and adipocytes. Although the cell sorting technologies of flow cytometry have been highly anticipated for the effective isolation of a specific subpopulation, some issues including the acquisition rates of target cells, the prevention of pathogen contamination, or the mechanical and thermodynamic damage to cells by the cell sorter should be cautiously evaluated before clinical use.

The MSC isolation was also attempted, using some surface epitopes, including CD13, CD29 (integrin β1), CD44 (hyaluronan receptor), CD73(SH3), CD90 (Thy-1), CD105 (Endoglin), CD166 (activated leukocyte cell adhesion molecule/ALCAM), PDGF receptor or Stro-1, all of which are highly expressed in the MSCs.[6,8] The combination with CD34 and CD45 (leukocyte common antigen/LCA), either of which is a marker of hematopoietic stem cells, could exclude the hematopoietic lineage from the MSCs. However, as the expression level of the markers in the MSCs was quite similar to those of fibroblastic cells that are also contained in bone marrow aspirates and that decrease the multipotency and self-renewal,[8] specific selection of the MSCs from such heterogenetic cell populations could not be sufficiently obtained even by flow cytometry or a magnetic cell sorting system.

Serum deprivation is one of the possible methods to concentrate the subpopulation possessing a high proliferation and differentiation potential from the heterogeneity of the MSCs.[9] When early-passage human MSCs were cultured in serum-free medium without cytokines or other supplements, a subpopulation of the cells was attached to the plates and survived for 2–4 weeks. Afterward, such cells began to proliferate in serum-containing medium, and prominently showed stem cell properties including long telomeres or a high expression of the octomer-binding transcription factor 4 (OCT-4). The findings suggested that such cells that possess a strong adherent ability and survive despite the harsh environments may show a high quality of stem cell properties.

On the basis of this hypothesis, we attempted to select some subpopulations of MSCs showing a high adhesiveness on the culture plates. For selection, the cell adhesiveness was adjusted by the coating of 2-methacryloyloxyethyl phosphorylcholine (MPC) polymers. The MPC polymers are designed with inspiration from cell membrane surface and well-known biocompatible polymers that can reduce protein adsorption or subsequent cell adhesion significantly.[10–12] On the basis of this fundamental biocompatibility, the MPC polymers have been used for preparing medical devices, for example, the surfaces of intravascular stents, intravascular guide wires, soft contact lenses, an artificial lung or artificial hip joint.[13–16] Some of these are already clinically available.

We examined the selectivity of MSCs using MPC polymer-coated plates and evaluated the proliferation ability or differentiation potential of the MPC polymer-selected subpopulation. Especially, we focused on the osteogenic and chondrogenic ability, because bone and cartilage tissue engineering using MSCs are highly desired for clinical applications.

MATERIALS AND METHODS

Preparation of MPC polymer-coated plates

Coating of the MPC polymer onto the polystyrene (PS) surface of the culture plates was performed by a simple dip-coating using MPC polymer solutions. The composition of MPC units was controlled by mixing poly(*n*-butyl methacrylate) (Poly(BMA)) and poly(MPC-*co*-BMA)(PMB30). These polymers were synthesized by a conventional radical polymerization. Poly(BMA) was a homopolymer of BMA without MPC unit (molecular weight = 4.0×10^5), and PMB30 was a copolymer composed of 30% of MPC units and 70% of BMA units (molecular weight = 6.0×10^5). In this study, each polymer was dissolved in a mixture of tetrahydrofran (THF) and ethanol (1:9 by volume) as solvents, and then poly(BMA) and PMB30 solutions were prepared (0.25 wt %). To control the MPC unit composition in the range between 0, 1, 2, 5, and 10% of MPC unit composition, these polymer mixtures in the solution were prepared. The dip-coating was carried out in the clean bench as follows: (i) 200 μL of the solution was poured into the each culture plates (ϕ 2.2 cm), (ii) the polymer solution was removed after 5 s, (iii) the coating was repeated and the resulting culture plate was dried over night, and (iv) the MPC polymer-coated culture plate was sterilized by UV irradiation for an adequate time. Therefore, the resulting MPC unit density on the plate was 0, 1, 2, 5, and 10% MPC unit composition.

Surface elemental analysis of the MPC polymer-coated PS plate was carried out by X-ray photoelectron spectroscopy (XPS, AXIS-His, Shimadzu/KRATOS, Kyoto, Japan). The X-ray source used for XPS measurements was Mg Ka source. The take-off angle of the photoelectrons was fixed

as 90°. At least five points of the sample were measured by XPS and these intensities were averaged before the following calculation. The surface compositions of the MPC units were calculated as follows. The ratio of signal intensity at 133 eV based on the phosphorus atom attributed to the MPC units over that at 285 eV based on the carbon atoms attributed methyl groups and methylene groups in both BMA and MPC units was determined. The calibration was carried out using the ratio obtained from the XPS spectra of both poly(BMA) and poly(MPC)-coated PS plate as 0% and 100% of MPC unit, respectively.

MSC preparation and selection by MPC polymer-coated plates

All procedures for the present experiments were approved by the ethics committee or institutional committee for animal research of the University of Tokyo Hospital (ethics permission #622). Figure 1(a) indicates the experimental design. Human MSCs were obtained from the femur of osteoarthritic patients who underwent total hip replacement at the University of Tokyo Hospital, after informed consent. Cells in bone marrow aspirates (100 μL/φ 2.2 cm dish) were seeded on MPC polymer-coated culture plates with various MPC unit compositions as 0–10%, and cultured using the hMSC bullet kit (Cambrex, East Ruatherford, NJ) in a 37°C/5% CO_2 incubator. Rat MSCs were collected from 6-week-old male Sprague-Dawley rats (Nisseizai, Tokyo, Japan). After the epiphyses of the tibias were removed, the marrow was flushed out by using a syringe filled with medium and filtered through a 70-μm nylon mesh. The obtained bone marrow materials (100 μL/φ 2.2 cm dish) were plated and cultured in the same manner as human MSCs.

The cells were harvested by treatment using trypsin-EDTA solution. After the cell harvest of the primary culture from the MPC polymer-coated plates, the cells were reseeded onto the conventional PS culture plates at a density of 5.0×10^3 cells/cm^2. Passages were performed when the cells were approaching confluence. The medium was changed three times per week. The cell numbers were counted by a haematocytometer, while the viability of the cells was checked by trypan blue staining. Cell proliferation was also colorimetrically measured by cell counting kit-8 (Dojin, Kumamoto, Japan), a week after cell seeding.

Flow cytometric analysis

Cells were harvested using trypsin-EDTA solution, centrifuged at 1500*g* for 5 min, and resuspended at 5×10^6 cells/mL in phosphate-buffered saline (PBS) containing 3% fetal bovine serum. Aliquots containing 10^5 cells were incubated with individual primary antibodies or control IgG for 30 min at room temperature. The cells were washed in PBS containing 3% fetal bovine serum and incubated with a fluorescent conjugated secondary antibody for 30 min at room temperature. Samples were analyzed using a FACS LSL II (BD, Franklin Lakes, NJ). The following monoclonal antibodies were used: mouse monoclonal antibodies against human CD29 (integrin β1, BD), human CD34 (Chemicon, Victoria, Australia), human CD44 (hyaluronan receptor, Ancell, Bayport, MN), human CD45 (LCA, Cymbus, Chandlers Ford, UK), human CD105 (Endoglin, Ancell), CD166 (ALCAM, Ancell), normal mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and fluorescein isothiocyanate (FITC)-conjugated rabbit antibody against mouse IgG (Santa Cruz Biotechnology).

Osteogenic and chondrogenic induction for MSCs

The osteogenic[1] or chondrogenic[17,18] differentiation was induced in MSCs according to previously reported procedures with some modifications. For the osteogenic differentiation, cells were seeded at 4.0×10^4 cells per 2.2-cm plates and maintained for 21 days in DMEM supplemented with 10% fetal bovine serum, 10 m*M* β-glycerophosphate, 100 n*M* Dexamethasone, and 50 μg/mL ascorbic acid-2-phosphate. For the chondrogenic differentiation, cells were seeded at 2×10^5 cells per 15 mL plastic centrifuge tube and maintained in 2 mL of serum-free a-MEM supplemented with 3500 μg/mL glucose, 6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 ng/mL selenite, 5.33 μg/mL linolate, 1.25 mg/mL bovine serum albumin, 10 ng/mL transforming growth factor-β3, 100 n*M* dexamethasone and 50 μg/mL ascorbic acid-2-phosphate. The cells were cultured under the chondrogenic status for 21 days. The medium was changed three times per week.

Total RNA extraction and real-time RT-PCR

The total RNA was isolated from MSC using the chaotropic Trizol method (Nippon-gene, Tokyo, Japan). The total mRNA (1 μg) was reverse transcribed using the Super Script reverse transcriptase with a random hexamer (Takara Shuzo, Shiga, Japan). The full-length or partial-length cDNA of the target genes, including the PCR amplicon sequences, was amplified by PCR, cloned into pCR-TOPO Zero II or pCR-TOPO II vectors (Invitrogen, Carlsbad, CA), and used as standard templates after linearization. The QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) was used, and the SYBR Green PCR amplification and real-time fluorescence detection were performed with an ABI 7700 Sequence Detection system (Foster City, CA). All reactions were run in quadruplicate. The sequences of the primers were 5′-CTCCT CGCTTTCCTTCCTCT-3′ and 5′-GTGCTAAAGGTGCCAA TGGT-3′ for COL1A1; 5′-GAGTCAAGGGTGATCGTGGT-3′ and 5′-CACCTTGGTCT CCAGAAGGA-3′ for COL2A1; 5′-AGGAATGCCT GTGTCTGCT T -3′ and 5′-ACAGGCC TACCCAAACATGA-3′ for COL10A1; 5′- GACCCTTGACC CCCACAAT-3′ and 5′- GCTCGTACTGCATGTCCCCT-3′ for ALP; 5′-CATG AGCGAGGG CACTCC-3′ and 5′-TCGCTTCAGGTCAGCCTTG-3′ for Sox9; 5′-GAAGGTGA AGGTCGGAGTCA-3′ and 5′-GAAGATGGTGATGGGAT TTC-3′ for GAPDH.

Enzyme activity for ALP

The enzyme activity was histochemically detected in the MSCs in which the osteogenic differentiation was induced.

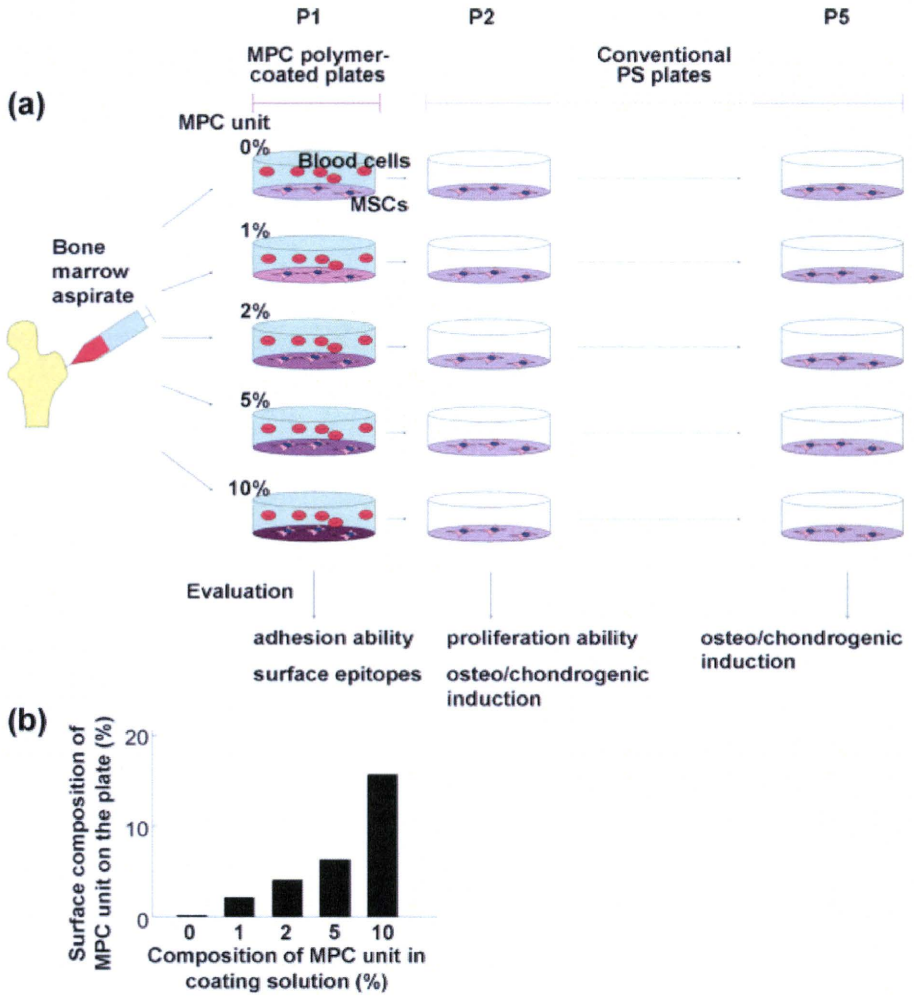

Figure 1. (a) The experimental design. Cells in bone marrow aspirates were seeded on MPC polymer-coated plates at the composition of 0–10% MPC unit, at passage 1, while the adhesion ability of MSCs to the MPC polymer-coated plates and the surface epitopes of MPC-selected cells were evaluated. Although cells were cultured on the MPC polymer-coated plates at passage 1, the cells were seeded onto the conventional PS plates thereafter. The proliferation of cells (passage 2) was measured by cell counting, while the differentiation potential for osteogenesis and chondrogenesis was examined at passages 2 and 5. (b) Relationship between MPC unit composition at the surface on PS plate after coating and that in polymer-coating solution. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

For ALP enzyme histochemistry, the cells were incubated with a mixture of 5 mg naphthol AS-BI phosphate (Sigma, St. Louis, MO) as a substrate and 18 mg of fast red violet LB salt (Sigma) diluted in 30 mL of 0.1 mol/L Tris-HCl buffer (pH 8.5). The images were taken by the digital camera, while the enzyme activity was quantitatively measured by histomorphometrical approaches using the software Scion Image alpha 4.0.3.2 (Scion, Frederick, MD).

RESULTS

Selection using MPC polymer-coated plates

Polymer coating of PS culture plate with the PMB30/poly(BMA) mixed solution was proceeded well, and the surface of the plate was covered with these polymers, completely. When the surface composition of MPC units on the plates was calculated from the XPS results, it was found that the MPC unit composition at the surface increased in parallel with that in the polymer mixed solution containing poly(BMA) and PMB30 used in a single dip coating as shown in Figure 1(b). We confirmed that the surface composition the MPC units could be controlled.

With these plates, we first selected some subpopulations of the MSCs according to the degrees of the adhesiveness on the culture plates coated with different compositions of the MPC unit. Human bone marrow aspirates (~0.1 mL) was seeded onto the culture plates with a 2.2 cm diameter coated with 0, 1, 2, 5, and 10%

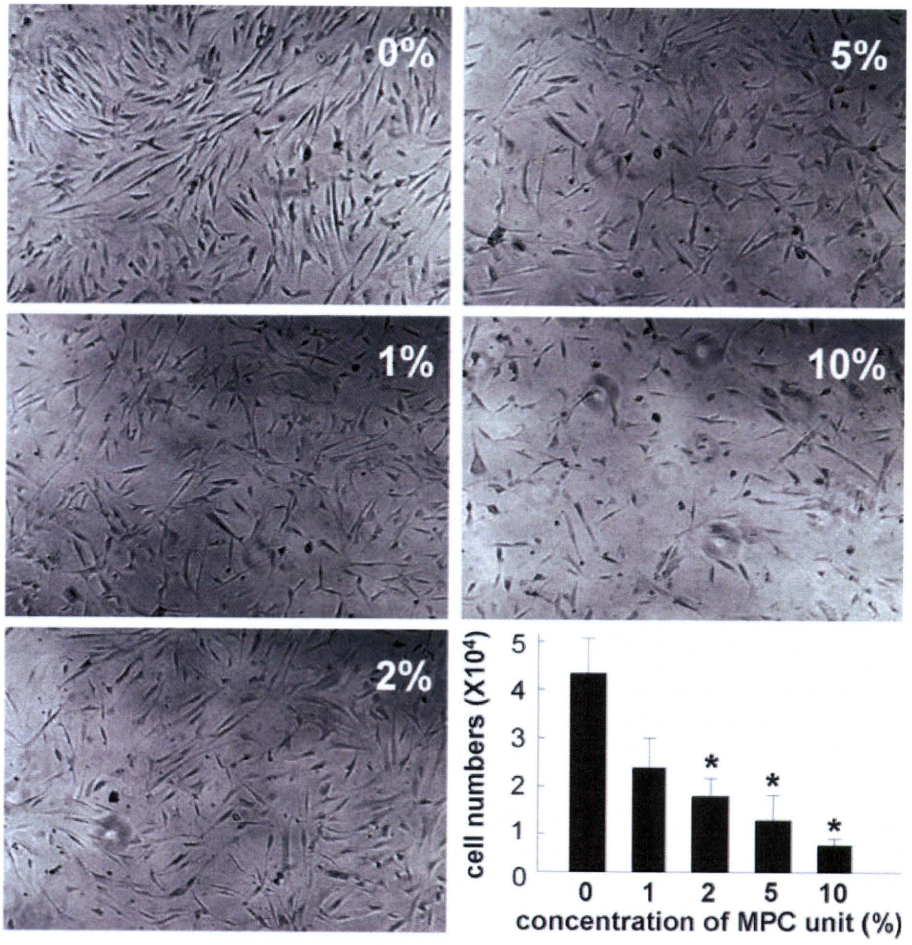

Figure 2. The adhesion of cells in human bone marrow aspirates onto the culture plates coated with MPC polymers with various compositions of MPC unit. The number of cells that were attached on the MPC polymer-coated plates at day 7 of the cell culture decreased according to the density of the MPC unit. All values are presented as mean plus standard deviation of five samples per group. Statistics were assessed using Dunnett's test (*$p < 0.01$ vs. 0% MPC unit composition).

MPC unit compositions. For 3 days, the number of adherent cells on the plate surface had plateaued on all plates. At 3 days, the medium was changed together with the floating cells and were replaced by another medium. The adherent cells continued to be cultured for four more days on the same MPC polymer-coated plates, and then were harvested for cell counting. The cells attached on the plate surface were observed to have a higher density on the dishes treated with a 0% MPC unit composition, compared with those of increasing the MPC unit composition, at 7 days (Fig. 2). The number of cells harvested from the plates had significantly decreased according to the increase in the density of the MPC polymer coating [Fig. 2 (graph)]. The cell numbers on the MPC polymer-coated dishes with 2% or 10% MPC units were approximately half or quarter of 0%, respectively.

To examine the proliferation ability of MPC polymer-selected MSCs, the cells harvested from each MPC polymer-coated plate were reseeded onto the conventional PS plates (φ 2.2 cm) with the same cell number of 1.9×10^4 in the second passage (passage 2), and then cultured for 7 days. The cells were equally proliferated during this period, while the total cell number after a 7 day-culture had not significantly changed among the cells derived from the different MPC polymer-coated plates [Fig. 3 (cell count)]. The result was represented by the experiment using the cell counting assay [Fig. 3 (cell counting assay)].

Surface epitopes of cells selected by MPC polymer-coated plates

We next examined the surface epitopes of the cells selected by the MPC polymer-coated plates (passage 1). It is known that CD29 (integrin β1), CD44 (hya-

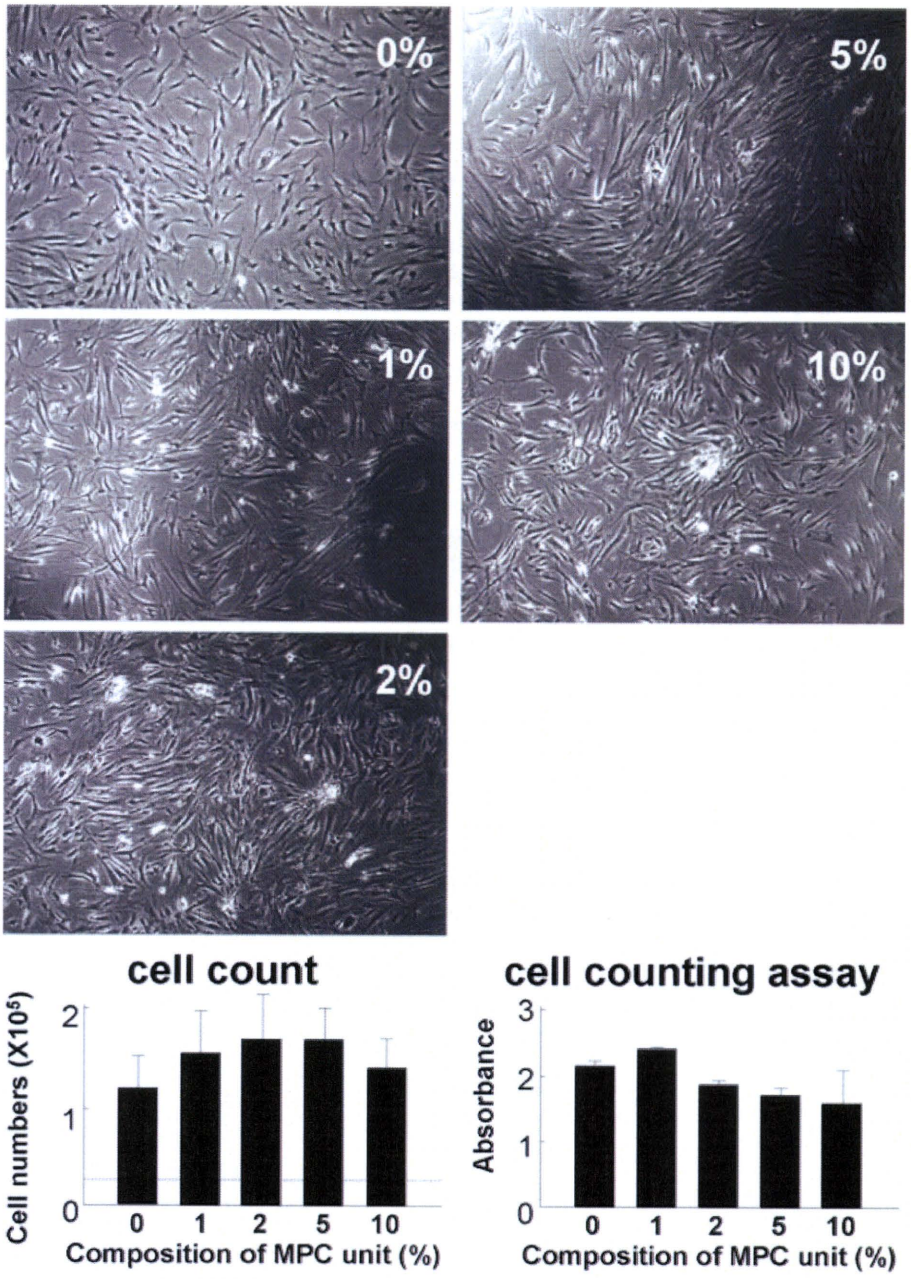

Figure 3. Proliferation of the cells that had been selected by the plate-coated MPC polymer with various MPC unit compositions. The cells cultured on the MPC polymer-coated plates were harvested and then reseeded onto the conventional PS plates. The numbers of human cells were counted at 7 days of culture (cell count). All values are presented as mean plus standard deviation of five samples per group. Statistics were assessed using Dunnett's test. No significant difference was seen among the proliferation of the cells harvested from each MPC polymer-coated plate (0–10% MPC unit composition). The dashed line indicats the number of cells originally seeded on the plate (1.9×10^3 cells). The result was represented by the experiment using the cell counting assay in the rat MSCs (cell counting assay). All values are presented as mean plus standard deviation of three measurements per group. No significant difference (Dunnett's test) was seen among each groups.

luronan receptor), CD105 (Endoglin) and CD166 (ALCAM) were expressed in MSC, but that CD34 and CD45 (LCA) were markers specific for heamatopoietic stem cells. Although the hematopoietic stem cell markers were negative in all cells selected by the plates coated with the 0, 1, 2, 5, or 10% of MPC unit

TABLE I
Expression of Surface Epitopes in MPC-Selected Cells

Surface Epitoptes	MPC 0%	MPC 1%	MPC 2%	MPC 5%	MPC 10%
CD29 (integrin β1)	++	++	+++	++	++
CD44 (Hyaluronan receptor)	++	++	++	++	++
CD105 (Endoglin)	+	+	+	+	+
CD166 (ALCAM)	+	+	+	+	+
CD34	−	−	−	−	−
CD45 (LCA)	−	−	−	−	−

composition, CD29, CD44, CD105, and CD166 were detectable in the cells of all MPC unit compositions. The levels of the MSC markers in the cells selected by the 1–10% MPC unit composition were almost similar to those in cells of 0% that corresponds to the control MSC, implying that the MPC polymer-selected cells belong to the category of MSC on the surface epitopes (Table I).

Osteogenic and chondrogenic potential of MPC polymer-selected cells

After the culture on the MPC polymer-coated plates (passage 1), the cells were cultured on the conventional PS culture plates for a long term with repeated passages. By passage 5, the cell numbers had expanded by approximately 1000-fold in the cells of each MPC unit composition (0–10%). Under the osteogenic condition, the cells selected by the MPC polymer coated-plates and cultured in the conventional PS plate ones for a single time (passage 2) more highly expressed the COL1A1 mRNA in the 2–5% MPC than in the 0%, but those by the 1 or 10% MPC polymer-coated plates did not show any significant increase in the COL1A1 expression. The promotion effects of the COL1A1 expression in 2% MPC unit composition continued even at passage 5, although the cells at passage 2 were more sensitive for the osteogenic differentiation than those at passage 5. ALP also peaks at 2-5% MPC unit composition for both passages, although no statistical difference of the ALP expression was detected in passage 2 [Fig. 4(a)]. The ALP enzyme activity was also significantly higher in 5% MPC unit composition than others at passage 2 [Fig. 4(b)].

The expression of the chondrocyte markers in the MPC polymer-selected cells under the chondrogenic conditions was also enhanced in the 2–5% MPC unit composition, as observed during osteogenesis. Responding to the chondrogenic induction, the cells began to express COL2A1, COL10A1, and Sox 9, and especially cells selected by the 2% MPC unit composition showed a prominent expression of all chondrocyte markers not only at passage 2, but even at passage 5 (Fig. 5).

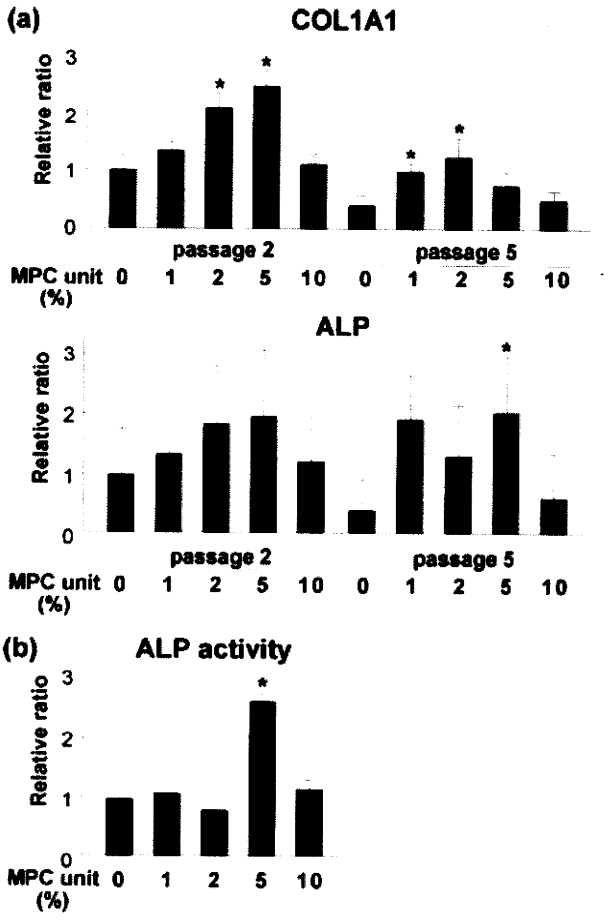

Figure 4. (a) Gene expression of COL1A1 and ALP in the osteogenic induction. Significant expression of COL1A1 gene was found in human MSCs selected by the MPC polymer-coated plates (2–5% unit composition) at passage 2, while the high expression level in the 5% MPC unit composition continued by passage 5. Also, in the ALP expression, the promotion effect was observed in 2–5% MPC unit composition, especially at passage 5. All values are presented as mean plus standard deviation of five samples per group. Statistics were assessed using Dunnett's test (*$p < 0.01$ vs. 0% MPC unit composition). (b) The enzyme activity for ALP in the osteogenic induction. The ALP enzyme activity was also significantly higher in 5% MPC unit composition than others in the rat MSCs at passage 2. All values are presented as mean plus standard deviation of three measurements per group. Statistics were assessed using Dunnett's test (*$p < 0.01$ vs. 0% MPC unit composition).

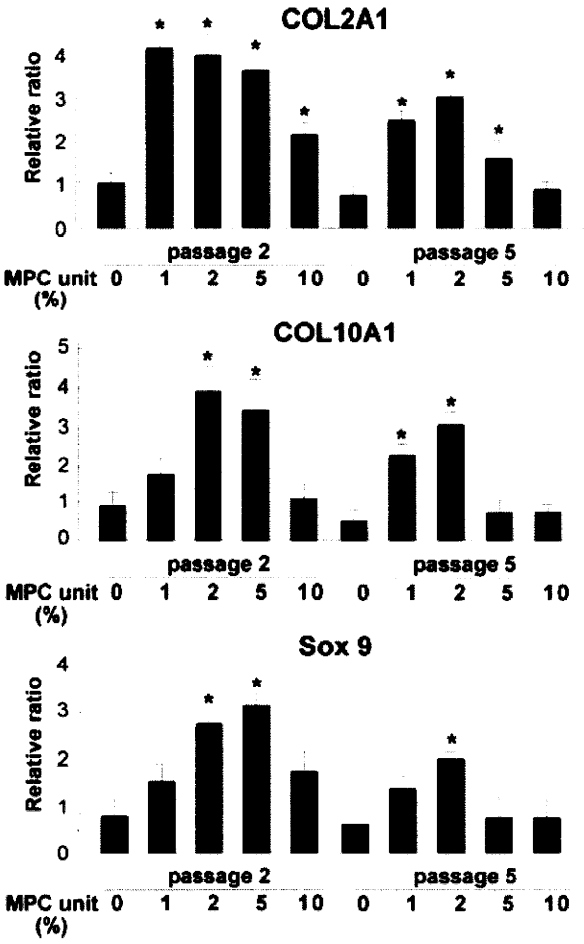

Figure 5. Gene expression of COL2A, COL10A1, and Sox9 during the chondrogenic induction. The expressions of COL2A1, COL10A1, and Sox9 genes peaked at 2–5% MPC unit composition not only at passage 2 but also at passage 5. All values are presented as mean plus standard deviation of five samples per group. Statistics were assessed using Dunnett's test (*$p < 0.01$ vs. 0% MPC unit composition).

DISCUSSION

The adhesion capacity seems to have some association with the cellular activities and functions. Specific adhesion to the laminin and type IV collagen coated on the surface of the culture dishes could select the myogenic cells of the embryonic mouse thigh from fibroblastic cells. Over a brief time period (10–20 min), myoblasts from the embryonic mouse thigh muscle had adhered faster to the laminin than did the fibroblasts from the same tissue, while the latter adhered faster to the fibronectin than the former.[19] Laminin-1 also enriched the osteoblast progenitor cells from rat calvarial cells when they were seeded on the culture wells coated with it. The laminin-1 inhibited cell attachment of the rat calvarial cells, but could select the highly osteogenic lineage according to the difference in the cell adhesiveness to that of the molecule.[20] Thus, through the selection of the cell adhesion to some molecules, a specific cell subpopulation that possesses a high differentiation potency would be concentrated from heterogeneity of the cell sources.

MSC expresses many adhesion-related molecules, like the integrin subunits α4, 5, 6, 8, 9, v/β1, 3, 5, ICAM-1, ALCAM, VCAM-1, SCF, fibronection, E-cadherin, and hyaluronan receptor[21–23] and can be bound to various ligands including laminin and E-cadherin to play biological roles through the cell-to-cell or cell-to-matrix contacts. As examples of the cell-to-cell contact with MSCs *in vivo*, homing functions for the hematopoietic cells of MSCs should be discussed. Through the cell-to-cell contacts with hematopoietic stem cells mediated by VCAM-1, fibronectin, SCF, E-cadherin, or ICAM-1, MSCs secrete extracellular matrix proteins, produce secreted/membrane-bound cytokines and regulate hematopoiesis.[22] MSCs are also recruited and adhered to the damaged tissues in order to participate in tissue repair. These cells can provide cell sources for tissue repair in bone, cartilage, and even skeletal muscle or myocardium that do not directly make contact with bone marrow. Once muscles are injured, the MSCs are delivered to the degenerative muscles from the circulation, are adhered to the lesion, take part in the regenerative process, and provide fully differentiated muscle fibers.[24] In the murine model of cardiac repair following ischemic injury, MSCs were mobilized from bone marrow, homed and generated cardiac myocytes. Among the adhesion molecules of the MSC such as integrin α4, 6, 8, 9, and β1, blockade of the integrin β1 by the neutralizing antibody reduced the total number of MSCs in the infracted myocardium, suggesting that MSCs utilized integrin β1 for cell adhesion to the myocardium and its regeneration.[23]

Thus, MSCs can be bound to various partners via many kinds of adhesion molecules to exert physiological and pathological functions. Although the adhesiveness to some ligands likely selects a cell subpopulation with a high differentiation potency of a certain lineage,[19,20] such a specific selection may have the risk to reduce the multipotency in MSCs. Therefore, we applied the selection system based not on the adhesiveness to specific molecules, but on the general adhesion ability to the MPC polymer-coated plates. As a result, we could enrich the cells to have a high potency of both osteogenesis and chondrogenesis from the crude MSCs.

It has yet remained unknown why the strength of the adhesion ability in MSCs could enhance not the proliferation rate of the cells, but the differential

potential for both osteogenesis and chondrogenesis. Speculating that such multipotent cells may show a stronger adhesion than fibroblastic cells in bone marrow, the MPC polymer-selection due to cell attachment could exclude the fibroblastic ones that possess a lower differentiation potential. This selection probably enriched the cells with high differentiation potential. It implied not that the MPC polymer-coated plates did not induce the phenotype changes in each cell, but that they purified the cell populations by the elimination of fibroblastic cells from the total populations of bone marrow adhesive cells. Therefore, the difference in osteogenic and chondrogenic ability was maintained during the repeated passaging, and the MPC polymer selection could improve cellular potential even after recultivation on PS plates. However, as we do not currently possess the methods to exactly distinguish MSCs from fibroblastic cells using cell surface epitopes, it may be hard to prove that the MPC selection could concentrate the multipotent MSCs from a mixture of the MSCs with fibroblast, by flow cytometry that can exactly exclude the hematopoietic lineage from the MSCs.

MSC can be differentiated into a variety of tissues including bone, cartilage, tendon, fat, heart, muscle or brain, *in vitro* and *in vivo*.[1,8] Autologous MSCs have advantages over embryonic stem cells, regarding the teratocarcinoma formation, immune rejection, or ethical problems. The cell sources have already been used for the treatment of osteogenesis imperfecta, bone/cartilage defects, myocardiac infarction, or skin ulcer.[25–28] Conversely, the MPC polymers have also been already applied in the clinical field for the surfaces of intravascular stents, intravascular guide wires, soft contact lenses, and the artificial lung, all of which were authorized by the United States Food and Drug Administration.[13,14] Thus, the biocompatible polymer is regarded to be approved for safe clinical use.

The MPC selection is as simple as to culture MSCs with MPC polymer-coated plates in the first passage, which would reduce the risks of contamination or mismanagement during the culture procedure. The improvement of the MSCs in purity and multipotency by the MPC polymer selection would provide promising technologies for the next generation-cell therapy that can be applied for more severe and other various diseases. The clinical application of the MPC polymer-selected MSCs is now underway.

References

1. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143–147.
2. Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, Sano M, Takahashi T, Hori S, Abe H, Hata J, Umezawa A, Ogawa S. Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 1999;103:697–705.
3. Kopen GC, Prockop DJ, Phinney DG. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. Proc Natl Acad Sci USA 1999;96:10711–10716.
4. Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luria EA, Ruadkow IA. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol 1974;2:83–92.
5. Sekiya I, Larson BL, Vuoristo JT, Cui JG, Prockop DJ. Adipogenic differentiation of human adult stem cells from bone marrow stroma (MSCs). J Bone Miner Res 2004;19:256–264.
6. Colter DC, Sekiya I, Prockop DJ. Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. Proc Natl Acad Sci USA 2001;98:7841–7845.
7. Smith JR, Pochampally R, Perry A, Hsu SC, Prockop DJ. Isolation of a highly clonogenic and multipotential subfraction of adult stem cells from bone marrow stroma. Stem Cells 2004;22:823–831.
8. Ishii M, Koike C, Igarashi A, Yamanaka K, Pan H, Higashi Y, Kawaguchi H, Sugiyama M, Kamata N, Iwata T, Matsubara T, Nakamura K, Kurihara H, Tsuji K, Kato Y. Molecular markers distinguish bone marrow mesenchymal stem cells from fibroblasts. Biochem Biophys Res Commun 2005;332: 297–303.
9. Pochampally RR, Smith JR, Ylostalo J, Prockop DJ. Serum deprivation of human marrow stromal cells (hMSCs) selects for a subpopulation of early progenitor cells with enhanced expression of OCT-4 and other embryonic genes. Blood 2004; 103:1647–1652.
10. Ueda H, Watanabe J, Konno T, Takai M, Saito A, Ishihara K. Asymmetrically functional surface properties on biocompatible phospholipid polymer membrane for bioartificial kidney. J Biomed Mater Res A 2006;77:19–27.
11. Sawada S, Iwasaki Y, Nakabayashi N, Ishihara K. Stress response of adherent cells on a polymer blend surface composed of a segmented polyurethane and MPC copolymers. J Biomed Mater Res A 2006;79:476–484.
12. Sibarani J, Takai M, Ishihara K. Surface modification on microfluidic devices with 2-methacryloyloxyethyl phosphorylcholine polymers for reducing unfavorable protein adsorption. Colloids Surf B Biointerfaces 2007;54:88–93.
13. Lewis AL, Tolhurst LA, Stratford PW. Analysis of a phosphorylcholine-based polymer coating on a coronary stent pre- and post-implantation. Biomaterials 2002;23:1697–1706.
14. Kihara S, Yamazaki K, Litwak KN, Litwak P, Kameneva MV, Ushiyama H, Tokuno T, Borzelleca DC, Umezu M, Tomioka J, Tagusari O, Akimoto T, Koyanagi H, Kurosawa H, Kormos RL, Griffith BP. In vivo evaluation of a MPC polymer coated continuous flow left ventricular assist system. Artif Organs 2003;27:188–192.
15. Moro T, Takatori Y, Ishihara K, Konno T, Takigawa Y, Matsushita T, Chung UI, Nakamura K, Kawaguchi H. Surface grafting of artificial joints with a biocompatible polymer for preventing periprosthetic osteolysis. Nat Mater 2004;3:829–836.
16. Goda T, Ishihara K. Soft contact lens biomaterials from bioinspired phospholipid polymers. Expert Rev Med Devices 2006;3:167–174.
17. Kato Y, Iwamoto M, Koike T, Suzuki F, Takano Y. Terminal differentiation and calcification in rabbit chondrocyte cultures grown in centrifuge tubes: Regulation by transforming growth factor beta and serum factors. Proc Natl Acad Sci USA 1988;85:9552–9556.

18. Ebisawa K, Hata K, Okada K, Kimata K, Ueda M, Torii S, Watanabe H. Ultrasound enhances transforming growth factor β-mediated chondrocyte differentiation of human mesenchymal stem cells. Tissue Eng 2004;10:921–929.
19. Kuhl U, Ocalan M, Timpl R, von der Mark K. Role of laminin and fibronectin in selecting myogenic versus fibrogenic cells from skeletal muscle cells in vitro. Dev Biol 1986;117:628–635.
20. Roche P, Rousselle P, Lissitzky JC, Delmas PD, Malaval L. Isoform-specific attachment of osteoprogenitors to laminins: Mapping to the short arms of laminin-1. Exp Cell Res 1999;250:465–474.
21. Conget PA, Minguell JJ. Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. J Cell Physiol 1999;181:67–73.
22. Hall BM, Gibson LF. Regulation of lymphoid and myeloid leukemic cell survival: Role of stromal cell adhesion molecules. Leuk Lymphoma 2004;45:35–48.
23. Ip JE, Wu Y, Huang J, Zhang L, Pratt RE, Dzau VJ. Mesenchymal stem cells utilize integrin beta 1 not CXC chemokine receptor 4 for myocardial migration and engraftment. Mol Biol Cell 2007;18:2873–2882.
24. Ferrari G, Cusella-De Angelis G, Coletta M, Paolucci E, Stornaiuolo A, Cossu G, Mavilio F. Muscle regeneration by bone marrow-derived myogenic progenitors. Science 1998;279:1528–1530.
25. Horwitz EM, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M, Sussman M, Orchard P, Marx JC, Pyeritz RE, Brenner MK. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med 1999;5:309–313.
26. Quarto R, Mastrogiacomo M, Cancedda R, Kutepov SM, Mukhachev V, Lavroukov A, Kon E, Marcacci M. Repair of large bone defects with the use of autologous bone marrow stromal cells. N Engl J Med 2001;344:385–386.
27. Vojtassak J, Danisovic L, Kubes M, Bakos D, Jarabek L, Ulicna M, Blasko M. Autologous biograft and mesenchymal stem cells in treatment of the diabetic foot. Neuro Endocrinol Lett 2006;27(Suppl 2):134–1371.
28. Fox JM, Chamberlain G, Ashton BA, Middleton J. Recent advances into the understanding of mesenchymal stem cell trafficking. Br J Haematol 2007;137:491–502.

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The optimization of porous polymeric scaffolds for chondrocyte/atelocollagen based tissue-engineered cartilage

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ABSTRACT

To broaden the clinical application of cartilage regenerative medicine, we should develop an implant-type tissue-engineered cartilage with firmness and 3-D structure. For that, we attempted to use a porous biodegradable polymer scaffold in the combination with atelocollagen hydrogel, and optimized the structure and composition of porous scaffold. We administered chondrocytes/atelocollagen mixture into the scaffolds with various kinds of porosities (80–95%) and pore sizes (0.3–2.0 mm), consisting of PLLA or related polymers (PDLA, PLA/CL and PLGA), and transplanted the constructs in the subcutaneous areas of nude mice. The constructs using scaffolds of excessively large pore sizes (>1 mm) broke out on the skin and impaired the host tissue. The scaffold with the porosity of 95% and pore size of 0.3 mm could effectively retain the cells/gel mixture and indicated a fair cartilage regeneration. Regarding the composition, the tissue-engineered cartilage was superior in PLGA and PLLA to that in PLA/CA and PDLA. The latter two showed the dense accumulation of macrophages, which may deteriorate the cartilage regeneration. Although PLGA or PLLA has been currently recommended for the scaffold of cartilage, the polymer for which biodegradation was exactly synchronized to the cartilage regeneration would improve the quality of the tissue-engineered cartilage.

1. Introduction

In the clinical approach of cartilage regenerative medicine, we often encounter large and extensive cartilage defects and dysfunction. They go far beyond the application range of current treatments, such as autologous chondrocyte transplantation which does not use a scaffold [1,2]. To overcome this situation, we attempted to apply the scaffold for making 'implant-type tissue-engineered cartilage' which has a firmness and 3-D structure.

To date, two types of scaffolds for cartilage tissue engineering have been used. The first is a hydrogel, while the second is a porous scaffold. Both types of scaffolds have some advantages and disadvantages. The former can provide a 3-D environment suitable for chondrocytes [3–6], but it does not have a mechanical strength. In contrast, the latter is hard enough to maintain the 3-D shape, although it does not provide a 3-D environment. Some of the groups and we have attempted to use both in order to overcome the disadvantages of each type [7–10]. In a previous study, we focused on the biological properties of these hydrogels, revealing that the atelocollagen favorably promoted the gene expression and the protein synthesis of cartilage matrices when it embedded the chondrocytes in a 3-D culture. In addition, the atelocollagen has already been used in a clinical application as a tissue for esthetic therapy [11], and is feasible for use as a scaffold of tissue engineering. Therefore, we have chosen the atelocollagen as the first line of hydrogel [12].

Regarding the porous scaffold, some groups attempted to use the fibrin sponge [13,14], hyaluronic acid polymer [15–17], collagen sponge [18,19] or biodegradable polymers [7–9,20,21]. Due to the firmness even when they are immersed in an aqueous environment, we regarded that the porous scaffolds made of biodegradable polymers were favorable for combination with the hydrogel [12]. However, these polymers generally have a hydrophobic property, which prevents infiltration of the highly viscose atelocollagen into the porous scaffolds of polymers and keeps the hydrogel materials within the porous scaffolds. Some optimization or improvement is

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needed to overcome these disadvantages of the biodegradable polymers, but there have been no systematic investigations on the structures and compositions of porous polymer scaffolds for use with atelocollagen. The purpose of the present study was to find the suitable structure and composition for the biodegradable porous polymer scaffolds.

To evaluate the structure, we used the polylactide (poly-L-lactide, PLLA, M_w 2 × 10^5) as the typical biodegradable polymer. We prepared various porous scaffolds with different structures using two procedures, i.e., the sugar leaching method (SLM) as the representative procedure [21] and the fused deposition modeling (FDM), one of the 3-dimensional modeling methods [20,22]. We examined the properties of these porous scaffolds regarding their mechanical strength, their affinity with the atelocollagen or the biocompatibility to the chondrocytes, and evaluated the biological characteristics of the tissue-engineered cartilage using these scaffolds.

However, PLLA has such a high crystallinity that it takes many years to be completely biodegraded [23–25]. As the next step of present study, we compared the PLLA scaffold with the lactide-related polymers that are more rapidly degraded than PLLA. The poly-D-lactide (PDLA) is isomeric with PLLA and is subject to

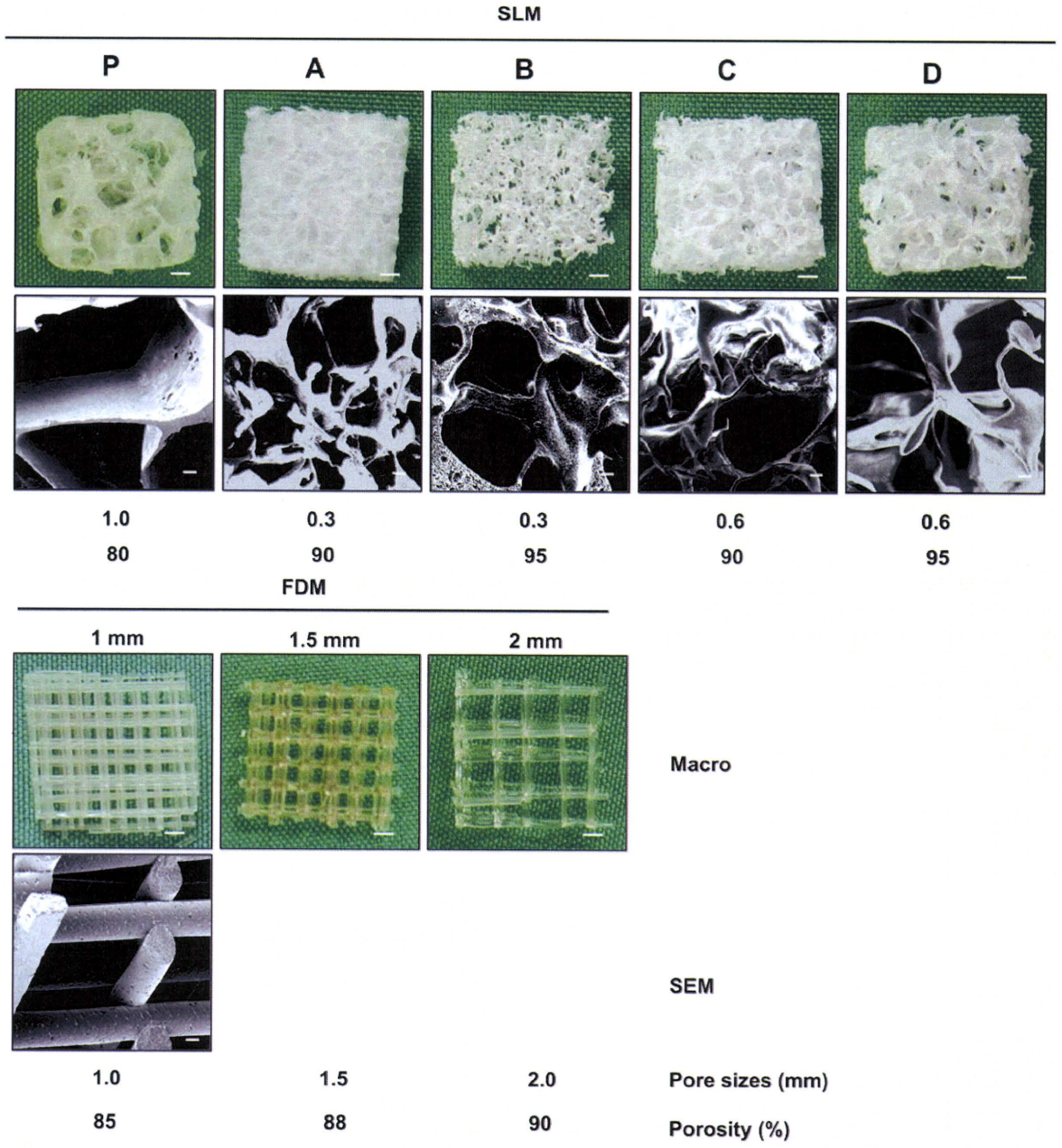

Fig. 1. Macroscopic (top) and SEM (bottom) images of porous scaffolds that had various structures. The SLM P (prototype) was made according to a previous paper [10]. We also prepared four kinds of scaffolds which possess structures with different pore sizes and porosities by the SLM (A–D). Three kinds of FDM scaffolds were manufactured, in which the width of the PLLA fibers was 1.0 mm and aperture size (pore size) of the scaffolds was 1.0 mm (1), 1.5 mm (1.5) and 2.0 mm (2). Pore size and porosity show the means of 6 samples. Bar; 1 mm and 100 μm for top and bottom, respectively.