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Figure captions

TABLE I Surface elemental composition (atom%) of CLPE, MPC polymer coated CLPE, and PMPC grafted CLPE

Figure 1. Scheme for the preparation of MPC polymer coated CLPE and PMPC grafted CLPE.

Figure 2. FT-IR/ATR spectra of untreated CLPE, MPC polymer coated CLPE, and PMPC grafted CLPE.

Figure 3. Cross-sectional TEM images of untreated CLPE, MPC polymer coated CLPE, and PMPC grafted CLPE. Bar: 200 nm.

Figure 4. Static-water contact angle of untreated CLPE, MPC polymer coated CLPE, and PMPC grafted CLPE. Bar: Standard deviations. *: Significant difference ($p < 0.001$) as compared to the untreated CLPE, **: significant difference ($p < 0.001$) as compared to the PMB30 coated CLPE.

Figure 5. Amount of BSA adsorbed on the surfaces of the untreated CLPE, MPC polymer coated CLPE, and PMPC grafted CLPE. Bar: Standard deviations. *: Significant difference ($p < 0.001$) as compared to the untreated CLPE.

Figure 6. Coefficients of dynamic friction of untreated CLPE, MPC polymer coated CLPE, and PMPC grafted CLPE. Bar: Standard deviations. *: Significant difference ($p < 0.005$) as compared to the untreated CLPE.

Figure 7. Coefficients of dynamic friction of the untreated CLPE, MPC polymer coated CLPE, and PMPC grafted CLPE as a function of loads in the ball-on-plate friction test. Bar: Standard deviations.

Figure 8. Weight change (volumetric wear) of the untreated CLPE, MPC polymer coated CLPE, and PMPC grafted CLPE in the hip joint simulator wear test. Bar: Standard deviations.

(n = 5)

Sample	C	O	N	P	Si
CLPE (untreated)	99.8 (0.3)**	0.2 (0.3)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
PMB30 coated CLPE	69.9 (1.0)	25.5 (0.6)	2.1 (0.2)	2.5 (0.3)	0.0 (0.0)
PMSi90 coated CLPE	60.5 (0.7)	30.4 (0.4)	4.1 (0.2)	4.0 (0.2)	1.0 (0.0)
PMPC grafted CLPE	58.0 (0.2)	31.5 (0.2)	5.2 (0.1)	5.3 (0.1)	0.0 (0.0)
PMPC*	57.9	31.6	5.3	5.3	0.0

*Theoretical elemental composition of PMPC.
**The standard deviation is in parentheses.

TABLE I Surface elemental composition (atom%) of CLPE, MPC polymer coated CLPE, and PMPC grafted CLPE
34x15mm (600 x 600 DPI)

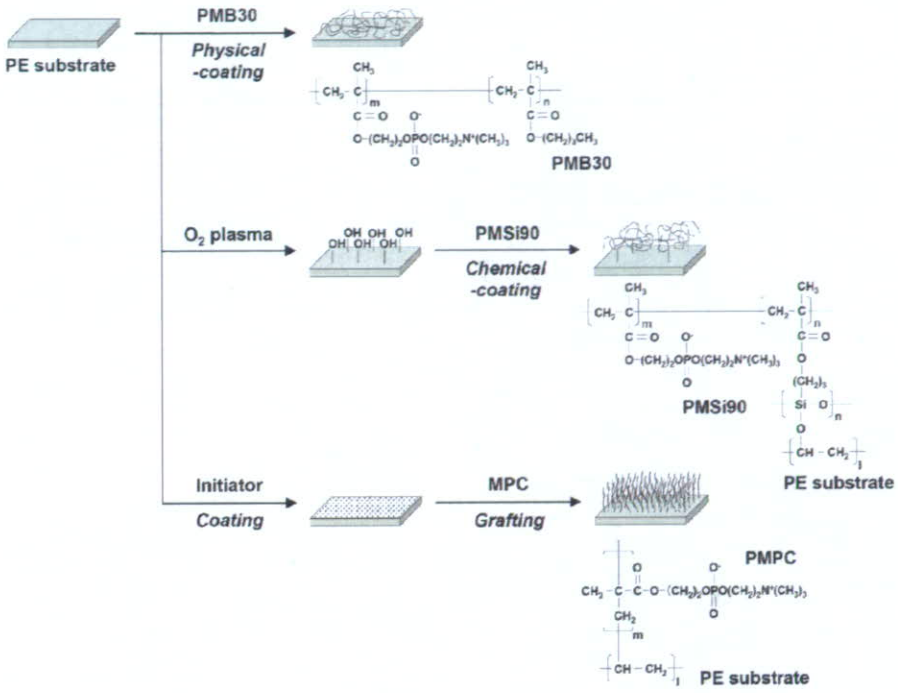

Figure 1. Scheme for the preparation of MPC polymer coated CLPE and PMPC grafted CLPE.

77x60mm (600 x 600 DPI)

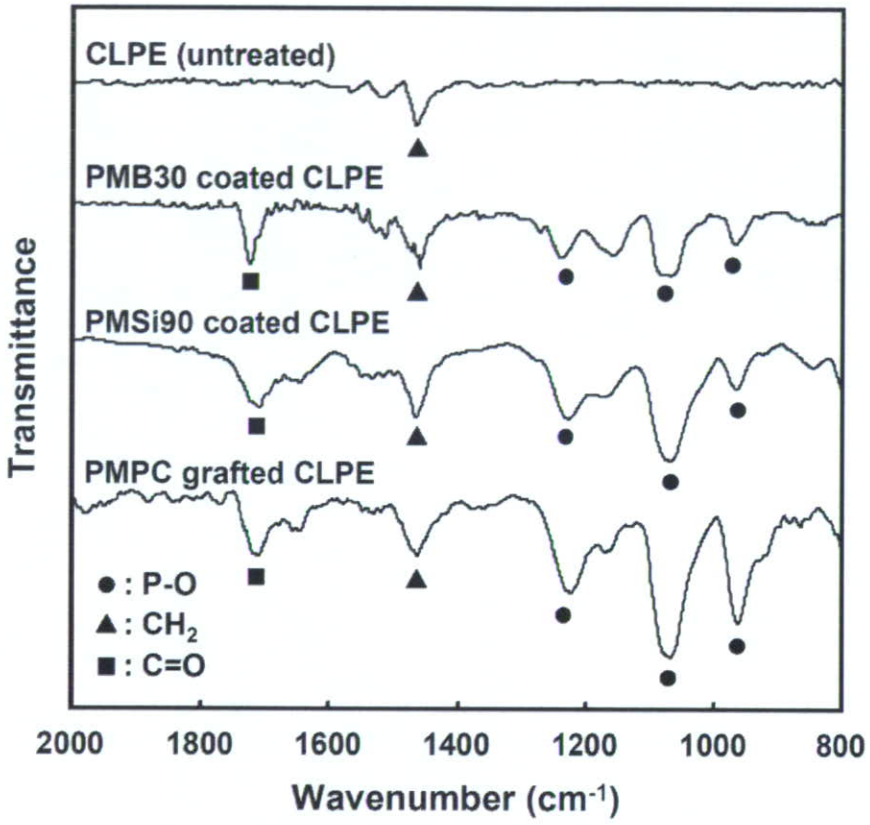

Figure 2. FT-IR/ATR spectra of untreated CLPE, MPC polymer coated CLPE, and PMPC grafted CLPE.
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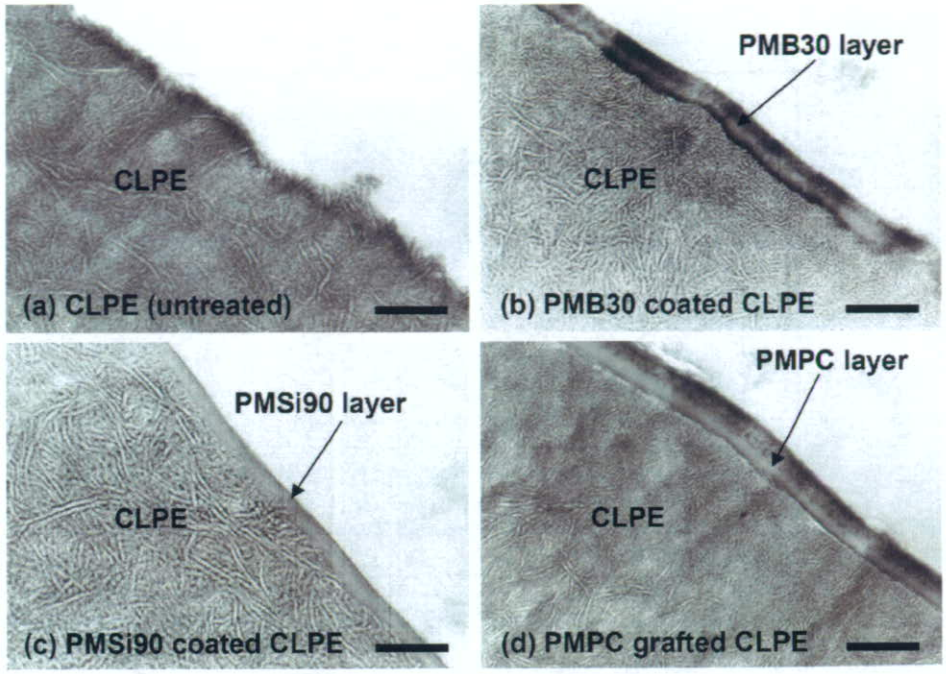
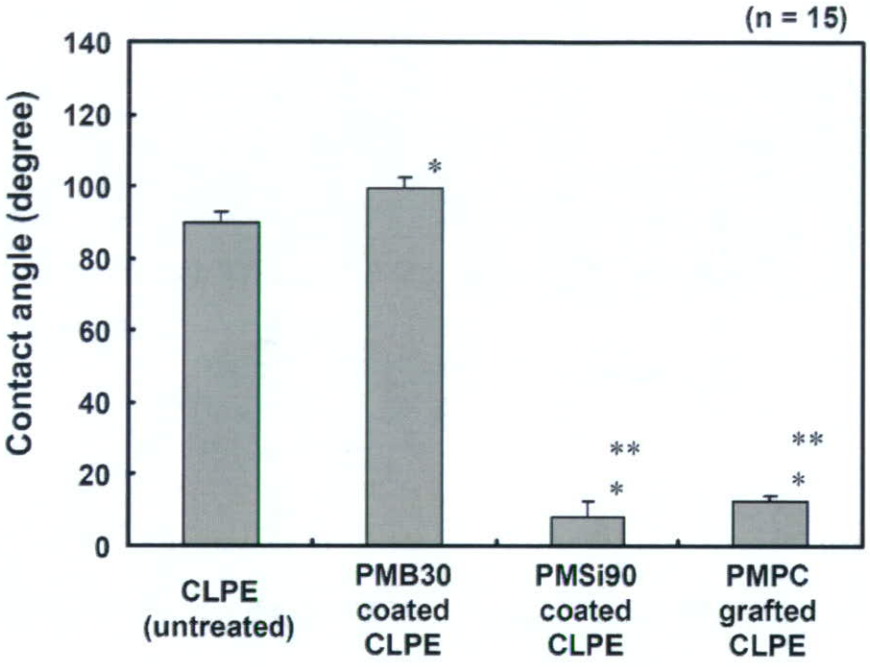


Figure 3. Cross-sectional TEM images of untreated CLPE, MPC polymer coated CLPE, and PMPC grafted CLPE. Bar: 200 nm.
49x35mm (600 x 600 DPI)



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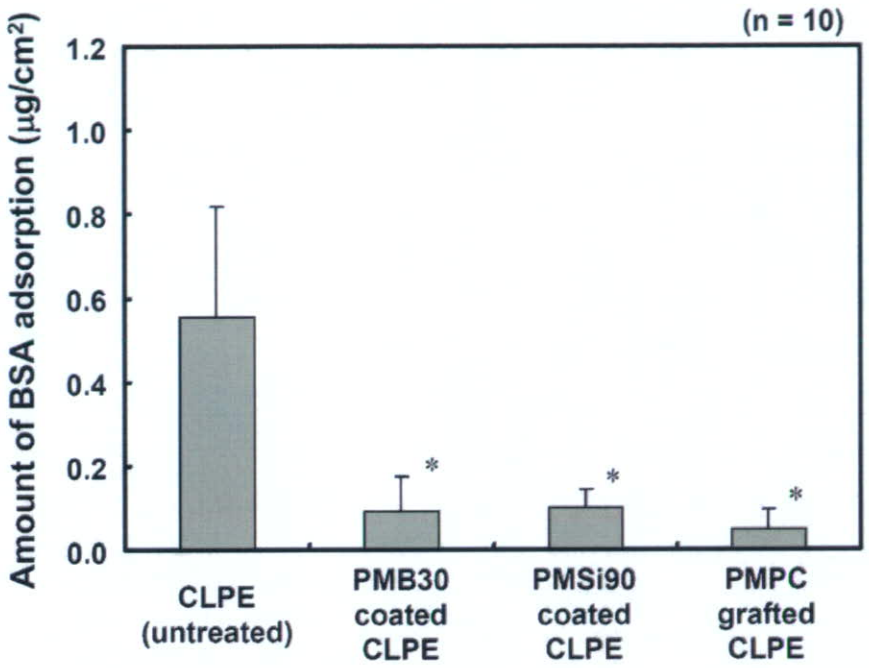
38x29mm (600 x 600 DPI)



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38x29mm (600 x 600 DPI)

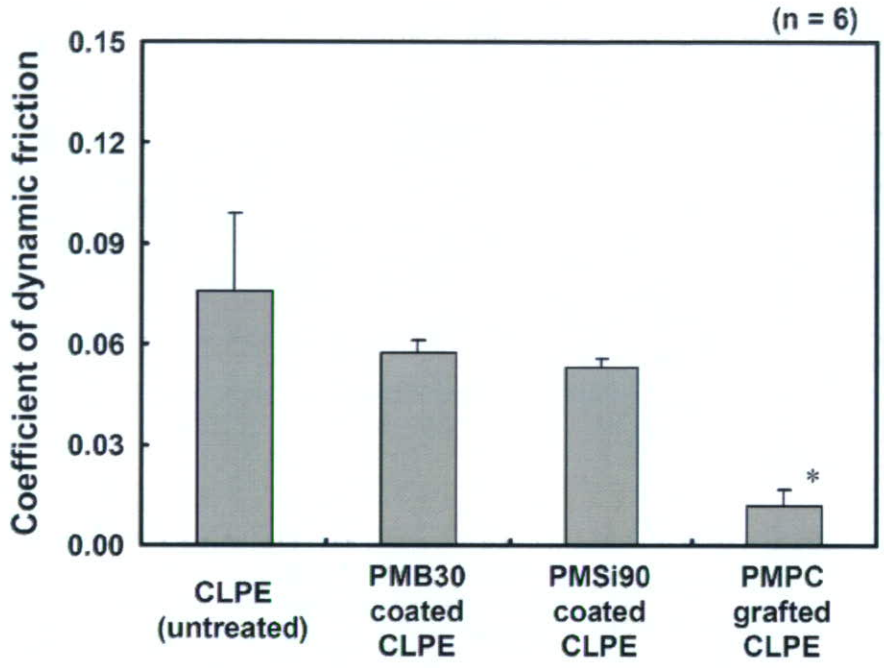

Figure 6. Coefficients of dynamic friction of untreated CLPE, MPC polymer coated CLPE, and PMPC grafted CLPE. Bar: Standard deviations. *: Significant difference ($p < 0.005$) as compared to the untreated CLPE.
38x29mm (600 x 600 DPI)

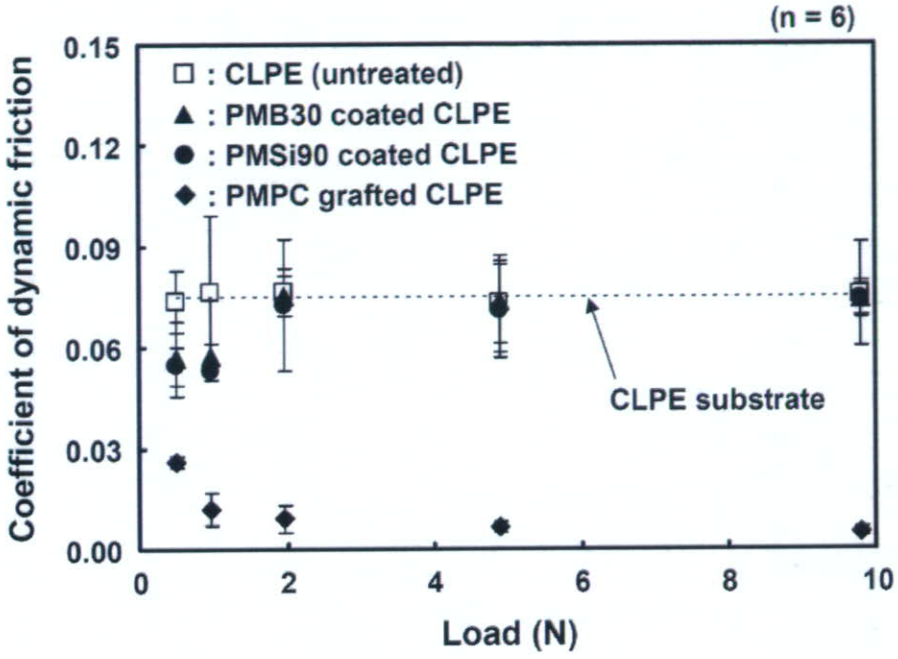

Figure 7. Coefficients of dynamic friction of the untreated CLPE, MPC polymer coated CLPE, and PMPC grafted CLPE as a function of loads in the ball-on-plate friction test. Bar: Standard deviations.
38x29mm (600 x 600 DPI)

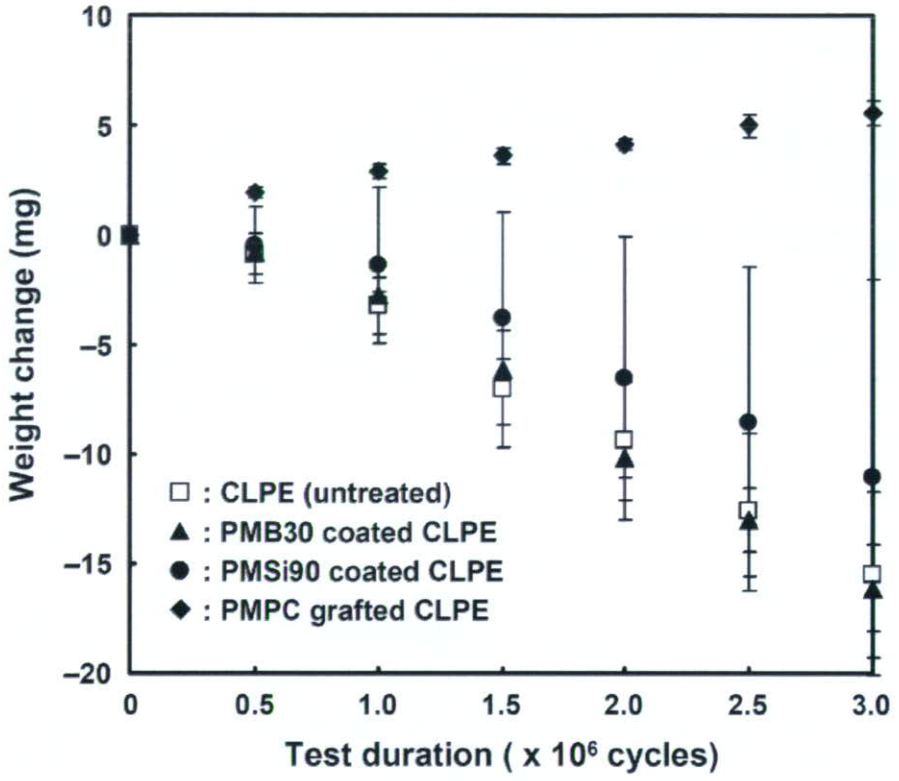

Figure 8. Weight change (volumetric wear) of the untreated CLPE, MPC polymer coated CLPE, and PMPC grafted CLPE in the hip joint simulator wear test. Bar: Standard deviations.
44x39mm (600 x 600 DPI)

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-selected MSCs. The number of MSCs that were attached to the MPC polymer-coated plates decreased with an increase in the density of MPC polymer (0-10%), while 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 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, 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.

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]

In order to isolate or enrich the subpopulation that preserves the self-renewal and the multipotentiality 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 to 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 in spite of the harsh environments may show a high quality of stem cell properties.

Based on 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) polymer. It consists of biocompatible

phospholipid polymers that resemble the phospholipids of biomembranes and that can reduce protein adsorption or subsequent cell adhesion.[10-12] This material was evaluated for use in medical devices, for example, the surfaces of intravascular stents, intravascular guide wires, soft contact lenses, an artificial lung or artificial joint, some of which are already clinically available.[13-16]

We examined the selectivity of MSCs using MPC polymer-coated plates and evaluated the proliferation ability or differentiation potential of the MPC-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 polysthylene (PS) surface of the culture plates was performed by a photoinduced polymerization technique that produced a covalent bond between the MPC and PS polymers, as previously described.[17] After the PS surface of the culture plates (φ 2.2 cm) was washed twice with methanol using an ultrasonicator for 30 min, the membrane was immersed in an acetone solution containing benzophenone (1.0 g/dL) for 30 sec. The plates were dried under a dark condition for 1 h in vacuo. The plates were filled with ethanol, while ultrasonication was then done for 10 min to detach the benzophenone from the PS surface. This washing process was repeated seven times. The MPC solution was prepared using degassed pure water. The MPC solution at the concentration of 0-10% was placed in a glass tube and argon gas was passed through the solution for 3 min to eliminate any oxygen. The MPC solution was then placed on the plate coated with benzophenone, and the photopolymerization on the PS surface of the culture plate was carried out using a 500 W ultra-high pressure mercury lamp at 60°C. The wavelength was

selected using a Toshiba D-35 filter (350950 nm). Afterwards, the plates were washed with water then hot ethanol (50°C), and dried in vacuo for 15 h.

MSC preparation and selection by MPC polymer-coated plates

All procedures for the present experiments were approved by the ethics committee of the University of Tokyo Hospital (ethics permission #622). Figure 1 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 at the concentration of 0-10%, and cultured using the hMSC bullet kit (Cambrex, East Ruatherford, NJ) in a 37°C/5% CO_2 incubator. 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 re-seeded onto the conventional PS culture plates at a density of 5000 cells/cm^2. Passages were performed when the cells were approaching confluence. The medium was changed three times/week. The cell numbers were counted by a haematocytometer, while the viability of the cells was checked by trypan blue staining.

Flow cytometric analysis

Cells were harvested using trypsin-EDTA solution, centrifuged at 1500 x g for 5 min, and resuspended at 5 x 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 or chondrogenic differentiation was induced in MSCs according to previously reported procedures[1] with some modifications. For the osteogenic differentiation, cells were seeded at 4 x 10^4 cells per 2.2 cm plates and
maintained for 21 days in DMEM supplemented with 10% fetal bovine serum, 10mM β-glycerophosphate, 100nM Dexamethasone, and 50 μg/mL ascorbic acid-2-phosphate. For the chondrogenic differentiation, cells were seeded at 2 x 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 nM 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/week.

Total RNA extraction and realtime RT-CR

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'-CTCCTCGCTTTCCTTCCTCT-3' and 5'-GTGCTAAAGGTGC CAATGGT-3' for COL1A1; 5'-GAGTCAAGGGTGATCGTGGT-3' and 5'-CACCTTGGTCT CCAGAAGGA-3' for COL2A1; 5'-AGGAATGCCT GTGTCTGCT T -3' and 5'-ACAGGCCTACCCAAACATGA-3' for COL10A1; 5'- GACCCTTGACCCCCACAAT-3' and 5'- GCTCGTACTGCATGTCCCCT-3' for ALP; 5'-CATG AGCGAGGG CACTCC-3' and 5'-TCGCTTCAGGTCAGCCTTG-3' for Sox9; 5'-GAAG GTGAAGGTCGGAGTCA-3' and 5'-GAAGATGGTGATGGGATTTC-3' for GAPDH.

RESULTS

Selection using MPC polymer-coated plates

We first selected some subpopulations of the MSCs according to the degrees of the adhesiveness on the culture plates coated with different concentrations of the MPC polymer. Human bone marrow aspirates (approximately 0.1 mL) was seeded onto the culture plates with a 2.2 cm diameter coated with 0, 1, 2, 5 and 10% MPC solutions. 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 4 more days on the same MPC polymer-coated plates, and then were harvested for cell counting.