Three-dimensional culture using human plasma-medium gel with fragmin/protamine microparticles for proliferation of various human cells

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Received: 27 January 2013 / Accepted: 29 July 2013 / Published online: 17 August 2013
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Abstract Fragmin/protamine microparticles (F/P MPs) have been used as carriers for the preservation and activation of cytokines in human plasma (HP)–Dulbecco’s modified Eagle’s medium (DMEM) gels. This study investigated a three-dimensional (3D) culture system using an HP–DMEM gel with 0.1 mg/mL F/P MPs and 5 ng/mL FGF-2 for the proliferation of human dermal fibroblast cells (DFCs), human microvascular endothelial cells (MVECs) and human coronary smooth muscle cells (SMCs), or 5 ng/mL interleukin (IL)-3/granulocyte-macrophage colony-stimulating factor (GM-CSF) for a human hematopoietic cell line (TF-1 cells). DFCs, MVECs, SMCs and TF-1 cells grew rapidly under 3D culture conditions using a low-concentration HP (2 %)-DMEM gel with F/P MPs and

FGF-2 (for DFCs, MVECs and SMCs) or IL-3/GM-CSF (for TF-1 cells) at doubling times of 22, 23, 25 and 18 h, respectively, without the use of animal serum, compared to under 2D culture conditions using low-concentration human serum (2 %)-DMEM with 5 ng/mL FGF-2 or IL-3/GM-CSF on F/P MP-coated plates at doubling times of approximately 26, 25, 40 and 20 h, respectively.

Keywords Cytokine carrier ·
Fragmin/protamine microparticles (F/P MPs) ·
Plasma–medium gel · 3D culture

Abbreviations

BMSCs Bone marrow stromal cells
DFCs Human dermal fibroblast cells

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DMEM	Dulbecco's modified Eagle's medium
ELISA	Enzyme-linked immunosorbent assay
F/P MPs	Fragmin/protamine microparticles
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HGF	Hepatocyte growth factor
HP	Human plasma
HS	Human serum
IL-3	Interleukin-3
KGF	Keratinocyte growth factor
MSCs	Mesenchymal stem cells
MVECs	Human microvascular endothelial cells
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
SMCs	Human coronary smooth muscle cells
TF-1 cells	Human hematopoietic cell line
2D	Two-dimensional
3D	Three-dimensional
TGF	Transforming growth factor
VEGF	Vascular endothelial growth factor

Introduction

Several different human cells such as dermal fibroblast cells (DFCs), microvascular endothelial cells (MVECs), vascular smooth muscle cells (SMCs) and hematopoietic cells (TF-1) are required for tissue engineering and regenerative medicine. Most protocols for the proliferation of human cells such as DFCs, MVECs, SMCs and TF-1 cells include high concentrations (approximately 10 %) of animal serum as a nutritional supplement (Kishimoto et al. 2009b). Some cell cultures include multiple doses of fetal bovine serum (FBS), which raises concerns over possible infections and immunological reactions due to medium-derived FBS proteins, sialic acid derivatives and other contaminants. Persistence of xenogeneic proteins in human mesenchymal stem cells (MSCs) proliferated using FBS was recently examined extensively (Martin et al. 2005; Spees et al. 2004). The results indicated that humoral immune responses against FBS proteins were observed in recipients after intravenous administration of autologous rat MSCs that had been proliferated using FBS. Therefore,

patients may experience problems during autologous cell-based therapies if non-autologous serum is used during cell culture. On the other hand, it is difficult to obtain large amounts of autologous serum from a patient for large-scale autologous cell culture.

Human plasma (HP) contains a high concentration of platelets, and various cytokines and bioactive proteins found in the α -granules of platelets augment tissue repair and regeneration processes (Marx 2001; Takikawa et al. 2011b, c). Platelets carry more than 20 cytokines, including platelet-derived growth factors (PDGFs), fibroblast growth factors (FGFs), hepatocyte growth factors (HGFs), transforming growth factors (TGFs), and vascular endothelial growth factors (VEGFs), most of which are known to bind to heparin. Recent studies suggest that cytokines in HP influence the viability of transferred cells, which may affect the proliferation and differentiation of adipocyte precursor cells. Furthermore, an HP-medium gel is formed by only the addition of HP to culture medium. Clinical studies have also documented the efficacy and safety of using HP in hard and soft tissue augmentation by stimulating and enhancing native repair and regeneration processes (Takikawa et al. 2011c; Bhanot and Alex 2002).

In addition to its well-known anticoagulant activity, heparin has been found to be associated with cytokines in various biological processes as well as involved in cell adhesion, recognition, migration and regulation of various enzymatic activities (Ishihara 1993; Ishihara and Ono 1998; Ruoslahti and Yamaguchi 1991; Salmivirta et al. 1996). Most cytokines present in HP are immobilized on the extracellular matrix by binding to heparin-like molecules (Ishihara 1993; Ishihara and Ono 1998; Ruoslahti and Yamaguchi 1991; Salmivirta et al. 1996). Protamine is a purified mixture of proteins obtained from fish sperm; it neutralizes both heparin and fragmin by forming a stable complex without anticoagulant activity (Pan et al. 1997). Protamine is also clinically used as an antidote to heparin by reversing the anticoagulant activity of heparin.

In previous studies, we used fragmin as a heparinoid and combined it with protamine to produce fragmin/protamine microparticles (F/P MPs) (Nakamura et al. 2009, 2010). F/P MPs efficiently bound to tissue culture plates, thereby retaining heparin-binding cytokines. Both exogenous and endogenous heparin-binding cytokines efficiently bound to F/P MPs, and their activities were maintained stable. Various human cells grew rapidly under two-dimensional (2D) culture

conditions using a low-concentration human serum (HS) (2 %)—Dulbecco's modified Eagle's medium (DMEM) with FGF-2 (for DFCs and MVECs) or IL-3/GM-CSF (for TF-1 cells) on F/P MP-coated tissue culture plates (Kishimoto et al. 2009a, b). In this study, the low-concentration HP (2 %)—medium gel with F/P MPs provided an extracellular matrix for the culture of different cell types by retaining endogenous and exogenous cytokines as well as forming a massive capsule with semipermeable properties, which allows the diffusion of medium components into cells and elimination of waste. Actually, human DFCs, MVECs, SMCs and TF-1 cells could grow faster in three-dimensional (3D) culture using the HP–DMEM gel with F/P MPs and adequate cytokines without the use of animal serum than those cultured under 2D culture condition using HS–DMEM with the cytokines on F/P MP-coated plates. The aim of this study was to establish an effective culture method for the proliferation of DFCs, MVECs, SMCs and TF-1 cells in 3D culture using the HP–DMEM gel with the cytokines compared to the previously reported proliferation in 2D culture (Kishimoto et al. 2009b).

Materials and methods

Preparation of F/P MPs

Fragmin/protamine microparticles (F/P MPs) were prepared as described previously (Mori et al. 2010; Takikawa et al. 2011a). In brief, protamine solution (10 mg/mL; Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) was added dropwise to fragmin solution (6.4 mg/mL; Kissei Pharmaceutical Co., Ltd., Tokyo, Japan) with vortexing for 2 min. A high yield of F/P MPs was obtained when 300 μ L protamine was mixed with 700 μ L fragmin (ratio = 3:7 v/v). To remove unreacted reagents, the mixture was centrifuged at $5,000\times g$ for 10 min at 4 °C using a high-speed centrifuge (MX-300, Tomy Seiko Co., Ltd, Tokyo, Japan). After removal of the supernatant, the pellets of MPs were resuspended in 1 mL Dulbecco's modified phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} . F/P MPs generated consisted of insoluble round complexes measuring approximately 0.5–3 μ m in diameter (average = ca. 1.5 μ m). A yield of >5 mg of dry F/P MPs was obtained from 1 mL of the resuspended F/P MPs solution.

For 2D culture, 24-well tissue culture plates (well area: 1.8 cm²) (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) were coated for 3 h at 4 °C with 0.3 mL of 0.35 mg/mL F/P MPs in PBS (Kishimoto et al. 2009a, b). Under this condition, approximately 30 μ g F/P MPs were immobilized to each well of 24-well tissue culture plates (Kishimoto et al. 2009a, b).

Scanning electron microscopy (SEM)

SEM was carried out using a JEOL JSM-6340F (JEOL Ltd., Tokyo, Japan) at 5 kV. SEM specimens of F/P MPs were prepared by coating a cover glass with a drop of 5 μ L water dispersion of the particles. The specimen was followed by gentle drying in a dry-box for overnight. To enhance conductivity of the specimen, osmium plasma coating was performed using the plasma multi-coater PMC-5000 (Meiwafosis Co., Ltd., Tokyo, Japan) after drying (Fig. 1B).

Preparation of HP

Human plasma (HP), platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared according to previously described methods (Marx 2001; Bhanot and Alex 2002). In brief, prior to the experiments on requirement of HP, PRP and PPP for cell culture and enzyme-linked immunosorbent assay (ELISA), 40 mL blood was drawn from volunteers into tubes containing 4 mL of 2 % sodium citrate (final 0.2 %) to avoid gelation. The tubes were centrifuged for 15 min at $400\times g$ (Table-Top Refrigerated Centrifuge 5800, Roter: RS-720, Kubota Corp., Tokyo, Japan), producing three layers: the PPP layer at the top, PRP layer in the middle, and erythrocytes at the bottom of the tube. The PRP and PPP layers contained HP. The upper 1 cm of the erythrocyte layer adjacent to the PRP layer was collected and centrifuged for 5 min at $1,400\times g$ to concentrate the platelets. HP, PRP and PPP were frozen at -80 °C until required (Takikawa et al. 2011c). Platelets in HP, PRP and PPP were counted under a microscope. The platelet concentration in the PRP layer ($79.6 \pm 10.2 \times 10^4/\mu\text{L}$, $n = 11$) was significantly higher than that in the HP ($18.2 \pm 3.6 \times 10^4/\mu\text{L}$, $n = 11$) and PPP layers ($3.6 \pm 0.6 \times 10^4/\mu\text{L}$, $n = 11$). All research protocols were submitted and approved by the Ethics Committee of the National Defense Medical College (Saitama, Japan) and blood donors were informed and signed written consent before participating in this study.

Fig. 1 Illustrations of 2D and 3D cell cultures (A). SEM observation of F/P MPs (B), and effect of sodium citrate and F/P MPs on HP–DMEM gelation (C). Five milliliter blood was drawn from volunteers into tubes containing various concentrations of sodium citrate to examine the HP–DMEM gelation (C; *left*). Furthermore, the inhibitory effect of various concentrations of F/P MPs added to HP–DMEM solution was examined to ensure HP–DMEM gelation (C; *right*). Data represent the mean $\pm$ SD for six measurements

ELISA to evaluate F/P MP binding of heparin-binding cytokines in HP, PRP and PPP

Enzyme-linked immunosorbent assay (ELISA) was performed to detect heparin-binding cytokines in HP, PRP and PPP and to evaluate the adsorption of those cytokines to F/P MPs. The indicated concentrations of F/P MPs were added to DMEM (Life Technologies Oriental Inc., Tokyo, Japan) with antibiotics (100 U/mL penicillin G and 100 μ g/mL streptomycin) and 2 % HP, PRP or PPP, which had been frozen and thawed at least twice, and incubated for 2 h at 37 °C on a rotary shaker. The mixtures were then centrifuged to remove F/P MPs. The levels of each cytokine in the diluted supernatants were measured using highly sensitive colorimetric sandwich ELISA kits (R&D

Systems, Inc., Minneapolis, MN, USA) for FGF-2, HGF, keratinocyte growth factor (KGF), VEGF, PDGF-AB, PDGF-BB and TGF- β 1, according to the manufacturer's instructions (Takikawa et al. 2011a, c).

Cell growth assays

Human DFCs, MVECs and aorta SMCs were purchased from Takara Biochemical Corp. (Otsu, Japan). The cells used in this study were between the fourth and eighth passages. TF-1 cells were purchased from CLS Cell Line Service GmbH (Eppenheim, Germany). DFCs, MVECs, SMCs and TF-1 cells were 2D cultured in DMEM supplemented with a low-concentration (2 %) of human serum (HS: SER019029 from human adults, Biopredic International Corp., Rennes,

France), antibiotics (100 U/mL penicillin G and 100 µg/mL streptomycin) and 5 ng/mL FGF-2 (Fiblast; Kaken Pharmaceutical Co., Ltd., Tokyo, Japan) (for DFCs, MVECs and SMCs) or 5 ng/mL IL-3/GM-CSF (GIBCO BRL., Life Technologies Inc., Carlsbad, CA, USA) (for TF-1 cells) on F/P MP-coated 24-well tissue culture plates (Kishimoto et al. 2009b) (Fig. 1A). After 2D culture, the medium was removed and rinsed with PBS, and the cells were released from the plates by treatment with 0.5 mL trypsin–EDTA solution (#59417C, Sigma Aldrich Japan LLC., Tokyo, Japan) at 37 °C for 5 min. The number of cells was then counted using a hemocytometer (Sigma Aldrich Japan) (Kishimoto et al. 2009a).

Prior to 3D culture, approximately 5.5 mL of HP was prepared from 10 mL blood drawn from volunteers into tubes containing 1 mL of 2 % sodium citrate (final 0.2 %). For 3D culture, the cells were suspended in 0.5 mL of a low-concentration HP (2 %)–DMEM solution with 0.1 mg/mL F/P MPs and the cytokines (5 ng/mL FGF-2 or IL-3/GM-CSF) (Fig. 1A), and those cells were then 3D cultured in 24-well tissue culture plates for the indicated time periods.

After 3D culture, the HP–DMEM gels were rinsed with PBS, and the cells were released from the gels by treatment with 0.5 mL of trypsin–EDTA solution at 37 °C for 15 min. Any remaining gel was dissolved by pipetting, and the cell numbers were counted using a hemocytometer.

Statistical analyses

Group means were compared by the unpaired Student's *t* test using Stat Mate III for Windows (ATMS Co., Ltd., Tokyo, Japan). $P \leq 0.05$ was considered statistically significant.

Results

Effect of sodium citrate and F/P MPs on low-concentration HP (2 %)–DMEM gelation

Five milliliter blood was drawn from volunteers into tubes containing 1.0, 0.5, 0.25, 0.125, 0.062, 0.031 and 0.015 mL of 2 % sodium citrate (final 0.4, 0.2, 0.1, 0.05, 0.025, 0.0125 and 0.0062 %, respectively) to examine HP–DMEM gelation (Fig. 1C, left). When 5 mL blood was drawn from volunteers into tubes

containing <0.015 mL of 2 % sodium citrate (final ≤ 0.006 %), the blood gelled immediately. On the contrary, when 5 mL blood was drawn into tubes containing >0.5 mL of 2 % sodium citrate (final ≥ 0.2 %), HP gelation was not observed (Fig. 1C, left). Prior to 3D cell culture using the HP–DMEM gel, approximately 2.6 mL of HP was prepared from 5 mL blood drawn from volunteers into tubes containing 0.5 mL of 2 % sodium citrate (final 0.2 %) to avoid the early blood gelation. The HP–DMEM gel could be generated by adding 0.1 mL the prepared HP and 50 µL of 2 % CaCl_2 (Nipro Corp., Osaka, Japan) to 5 mL DMEM and by incubating at 37 °C for 30 min.

The addition of high concentrations of F/P MPs (≥ 0.2 mg/mL) in the HP–DMEM solution also resulted in inhibition of HP–DMEM gelation (Fig. 1C, right). Therefore, 0.1 mg/mL of F/P MPs was added to HP–DMEM solution for 3D cell culture.

Adsorption of cytokines to F/P MPs

FGF-2, HGF, KGF, VEGF, PDGF-AB, PDGF-BB and TGF- β 1 present in HP, PRP and PPP are all known to specifically bind to heparinoids (Ishihara 1993; Ishihara and Ono 1998; Ruoslahti and Yamaguchi 1991; Salmivirta et al. 1996). The cytokines in frozen and thawed HP, PRP and PPP were measured by ELISA, as described above. The amounts of FGF-2, HGF, KGF, VEGF, PDGF-AB, PDGF-BB and TGF- β 1 in 2 % HP, PRP and PPP are summarized in Table 1. The indicated amounts of F/P MPs were added to 1 mL DMEM containing 2 % HP, PRP and PPP, incubated at 37 °C for 2 h, and centrifuged to separate the cytokine-containing F/P MPs. All cytokines in the supernatants were significantly decreased in a concentration-dependent manner, indicating that a significant amount of the cytokines was adsorbed to F/P MPs (Table 1).

Requirements of HP, PRP, PPP or HS for cultures of DFCs, MVECs, SMCs and TF-1 cells

DFCs, MVECs, SMCs and TF-1 cells growth was examined by 3-day cultures in DMEM with F/P MPs and FGF-2 (for DFCs, MVECs and SMCs) or IL-3/GM-CSF (for TF-1 cells), at an indicated concentration of HP, PRP, PPP or HS. Maximal cell growth was achieved with HP (≥ 2 %), PRP (≥ 1 %), PPP (≥ 4 %) and HS (≥ 4 %) DMEM (Figs. 2A–D). The results

Table 1 Concentrations of free cytokines in 2 % HP, PRP and PPP in the presence of F/P MPs

2 % HP	F/P MPs (mg/mL)				
Concentration	0	0.05	0.1	0.2	0.5
*FGF-2 (pg/mL)	0.07	ND	ND	ND	ND
*HGF (pg/mL)	2.9	0.5	ND	ND	ND
*KGF (pg/mL)	2.2	1.4	0.3	0.2	0.2
*VEGF (pg/mL)	3.6	1.4	0.2	0.2	0.3
*PDGF-AB (pg/mL)	1,220	530	170	150	140
*PDGF-BB (pg/mL)	18.8	7.8	3.6	2.6	1.5
*TGF- β 1 (pg/mL)	10.2	8.4	3.6	2.2	2.0
2 % PRP	F/P MPs (mg/mL)				
Concentration	0	0.05	0.1	0.2	0.4
*FGF-2 (pg/mL)	0.15	0.03	ND	ND	ND
*HGF (pg/mL)	4.4	0.8	ND	ND	ND
*KGF (pg/mL)	3.2	2.4	2.0	1.7	1.1
*VEGF (pg/mL)	7.0	2.1	1.2	1.0	1.1
*PDGF-AB (pg/mL)	3,400	1,600	580	245	121
*PDGF-BB (pg/mL)	48.0	17.0	11.0	8.0	5.0
*TGF- β 1 (pg/mL)	24	17	10	8	8
2 % PPP	F/P MPs (mg/mL)				
Concentration	0	0.05	0.1	0.2	0.5
*FGF-2 (pg/mL)	0.02	ND	ND	ND	ND
*HGF (pg/mL)	0.2	ND	ND	ND	ND
*KGF (pg/mL)	0.3	0.06	ND	ND	ND
*VEGF (pg/mL)	0.4	0.04	ND	ND	ND
*PDGF-AB (pg/mL)	300	100	30	20	20
*PDGF-BB (pg/mL)	5.0	1.2	0.8	0.6	0.5
*TGF- β 1 (pg/mL)	1.6	0.7	0.5	0.3	0.3

* Data were re-calculated as concentrations of each cytokine in the supernatants of 2 % HP, PRP, and PPP

showed that PRP is the most effective at enhancing cell proliferation, but it is difficult to produce a DMEM gel for 3D cultures using 1 % PRP. Furthermore, the yield of HP in volume from human blood is five-fold higher than that of PRP. Therefore, we decided to use the low-concentration HP (2 %)-DMEM gel with F/P MPs and those cytokines for 3D culture.

Growth of DFCs, MVECs, SMCs and TF-1 cells in 2D and 3D cultures using a low-concentration HP-medium gel with F/P MPs and cytokines

Growth of DFCs, MVECs, SMCs and TF-1 cells was stimulated in 2D culture using low-concentration HS

(2 %)-DMEM with FGF-2 (for DFCs, MVECs and SMCs) or IL-3/GM-CSF (for TF-1 cells) on F/P MP-coated plates, with doubling times of approximately 26, 25, 40 and 20 h, respectively. Furthermore, the growth of DFCs, MVECs, SMCs and TF-1 cells was stimulated in 3D culture using the HP-DMEM gel with F/P MPs and FGF-2 (for DFCs, MVECs and SMCs) or IL-3/GM-CSF (for TF-1 cells), with shorter doubling times of 22, 23, 25 and 18 h, respectively (Figs. 3, 4, 5, 6). All cells exhibited better growth under 3D conditions in HP-DMEM gels with F/P MPs and adequate cytokines. Microscope observation revealed little dead cells in the 3D-cultured cells. We evaluated that the cell survival as well as cell growth in the 3D-culturing were better than those in 2D-culturing. The yield of cells cultured for 4 days under 3D conditions in the HP-DMEM gel with F/P MPs and those cytokines was ≥ 20 % higher than the yield of cells in 2D culture on F/P MP-coated plates using HS-DMEM with those cytokines and on uncoated tissue culture plates using high-concentration HS (10 %)-DMEM (Figs. 3, 4, 5, 6). In 2D culture, DFCs, MVECs, and SMCs adhered to plates and exhibited a spindle shape at 5 h, whereas the cells grown in 3D culture using the HP-DMEM gel with F/P MPs and FGF-2 exhibited a thin spindle shape and generated a 3D network within 2 days (Figs. 3, 4, 5, 6).

When DFCs (about 100,000 cells/0.5 mL) were cultured under the 3D condition for >3 days at a high cell density using the HP-DMEM gel with F/P MPs and FGF-2, the culture gels containing only DFCs contracted after the formation of granulation-like tissue. Volume of the 3D culture using the HP-DMEM gel with F/P MPs and FGF-2 (about 0.9 cm³) was not changed during the first 2 days and decreased from 0.9 to 0.8 cm³ on day 3. Furthermore, the volume of the 3D culture using the HP-DMEM gel with F/P MPs and FGF-2 was decreased to 0.5 cm³ after 5 days-culturing. In contrast, only minor stimulation of MVEC growth and partial capillary-like tissue formation was observed in 3D culture (Fig. 4) and there was little contraction.

Discussion

Patients may experience problems while undergoing autologous cell-based therapies if non-autologous

Fig. 2 Concentration of HP, PRP, PPP or HS for the proliferation of various human cells. DFC (A), MVEC (B), SMC (C) and TF-1 (D) cell growth was examined by 3-day cultures in DMEM with F/P MPs and adequate cytokines using the indicated concentrations of HP (*filled circle*), PRP (*filled triangle*), PPP (*filled square*) or HS (*open circle*). Data represent the mean $\pm$ SD for six measurements

serum is used during cell culture. On the other hand, it is difficult to obtain large volumes of autologous serum from a patient. We previously produced F/P MPs as carriers for the controlled release of heparin-binding cytokines such as FGF-2, IL-3 and GM-CSF (Nakamura et al. 2009; Kishimoto et al. 2009b). F/P MPs can be stably coated on plastic and glass surfaces. Our previous study demonstrated the utility of an F/P MP coating as a heparin-binding cytokine-immobilizing substratum to stimulate the growth of bone marrow stromal cells (BMSCs) (Kishimoto et al. 2009a, b). Furthermore, we reported that DFCs, MVECs and TF-1 cells could be proliferated in 2D culture using HS-DMEM with adequate cytokines on F/P MP-coated plates. In this study, we were able to proliferate DFCs,

MVECs, SMCs and TF-1 cells and observed a higher growth rate in 3D cell culture using the HP-DMEM gel with F/P MPs and the cytokines than in 2D cell culture, as reported previously (Kishimoto et al. 2009b). We observed capillary-like tissue formation as well as rapid cell growth when MVECs were proliferated in 3D culture using the HP-DMEM gel with F/P MPs and FGF-2. These results suggest that the proliferation of those cell types may be possible under 3D conditions using the autologous HP-DMEM gel with F/P MPs and adequate cytokines.

We previously reported that F/P MPs, measuring 0.5–3 μ m in diameter (average = approximately 1.5 μ m), can be stably coated on plastic and glass surfaces (Kishimoto et al. 2009a, b) and that DFCs and

Fig. 3 Proliferation of DFCs in 2D and 3D cultures. *Left panels* DFCs (*upper figure*) were cultured in a 3D system using the HP-DMEM gel with F/P MPs and FGF-2 (*filled square*), with F/P MPs alone (*open triangle*), FGF-2 alone (*open square*) and no supplementation (*filled circle*). *Right panels* DFCs (*upper figure*) were cultured in a 2D system using the HS-DMEM on F/P MP-coated plates (*open triangle*) with FGF-2 (*filled square*), on uncoated plates (*filled circle*), with FGF-2 alone (*open square*)

and using high-concentration HS (10 %)–DMEM on uncoated plates (*open circle*). Proliferation of DFCs was measured for 4 days. Data represent the mean $\pm$ SD. *Lower panels* show photomicrographs of 3D and 2D cultures of DFCs after 3 days of culture to illustrate their morphologies. The differences between the experimental groups in 3D culture (HP-DMEM gel with F/P MPs and FGF-2) and in 2D culture (HS-DMEM on F/P MP-coated plates with FGF-2) were significant (** $P < 0.01$ ($n = 6$))

MVECs could be proliferated in DMEM using low-concentration serum (as low as 2 % HS or FBS) on adequate cytokine-immobilized F/P MP-coated plates (Kishimoto et al. 2009b). However, DFCs, MVECs and SMCs did not adhere or grow well on plates coated with a high density of F/P MPs (data not shown) because of the hydrogel-like property of the coating matrix. The 3D cell culture system described in this article included cells combined with a HP-DMEM gel

with F/P MPs and adequate cytokines; each cell type tested grew faster in 3D culture than in 2D culture. Furthermore, the 3D culture methods required no animal serum because low-concentration (2 %) of autologous plasma was sufficient.

Platelets are a natural source of cytokines such as PDGFs, HGFs, TGFs, FGFs and VEGFs, which are stored in the α -granules of platelets. Platelets are activated by factors such as thrombin or calcium to

Fig. 4 Proliferation of MVECs in 2D and 3D cultures. *Left panels* MVECs (*upper figure*) were cultured in a 3D system using the HP-DMEM gel with F/P MPs and FGF-2 (*filled square*), with F/P MPs alone (*open triangle*), FGF-2 alone (*open square*) and no supplementation (*filled square*). *Right panels* MVECs (*upper figure*) were cultured in a 2D system using the HS-DMEM on F/P MP-coated plates (*open triangle*) with FGF-2 (*filled square*), on uncoated plates (*filled circle*), with FGF-2 alone (*open square*) and using high-concentration HS (10 %)-

DMEM on uncoated plates (*open circle*). Proliferation of MVECs was measured for 4 days. Data represent the mean $\pm$ SD. *Lower panels* show photomicrographs of 3D and 2D cultures of MVECs after 3 days of culture to illustrate their morphologies. The differences between the experimental groups in 3D culture (HP-DMEM gel with F/P MPs and FGF-2) and in 2D culture (HS-DMEM on F/P MP-coated plates with FGF-2) were significant (* $P < 0.05$ ($n = 6$))

release these cytokines from the α -granules (Marx 2001; Takikawa et al. 2011c; Bhanot and Alex 2002). Integration of a wide variety of cytokines may be useful in tissue engineering strategies to mimic the natural environments of tissue formation (Chen et al. 2001). Any treatment that aims to mimic the crucial aspects of the natural biological process should not be

limited to the provision of a single cytokine, and multiple cytokines should be released at an optimized ratio in terms of the physiological dose and the specific spatiotemporal pattern (Takikawa et al. 2011a, b, c; Chen et al. 2001). In this study, we demonstrated that HGF, KGF, VEGF, PDGF and TGF in HP can be efficiently immobilized on F/P MPs. However, we

Fig. 5 Proliferation of SMCs in 2D and 3D cultures. *Left panels* (upper figure) were cultured in a 3D system using the HP-DMEM gel with F/P MPs and FGF-2 (filled square), with F/P MPs alone (open triangle), FGF-2 alone (open square) and no supplementation (filled circle). *Right panels* SMCs (upper figure) were cultured in a 2D system using the HS-DMEM on F/P MP-coated plates (open triangle) with FGF-2 (filled square), on uncoated plates (filled circle), with FGF-2 alone (open square) and

using high-concentration HS (10 %)-DMEM on uncoated plates (open circle). Proliferation of SMCs was measured for 4 days. Data represent the mean $\pm$ SD. *Lower panels* show photomicrographs of 3D and 2D cultures of SMCs after 3 days of culture to illustrate their morphologies. The differences between the experimental groups in 3D culture (HP-DMEM gel with F/P MPs and FGF-2) and in 2D culture (high-concentration HS (10 %)-DMEM on uncoated plates) were significant (** $P < 0.01$ ($n = 6$)))

added 5 ng/mL FGF-2 to the HP-DMEM gel because FGF-2 in 2 % HP was very low (0.07 pg/mL; Table 1). Although the content of IL-3/GM-CSF in HP was not measured in this study, those cytokines may be very low because of requirement of exogenous IL-3/GM-CSF for growth of TF-1 cells. The F/P MPs combined with the HP-DMEM gel used in this study may adsorb various heparin-binding substances from

HP and exogenously added cytokines, and the adsorbed substances immobilized within the HP-DMEM gel may stimulate cell proliferation. Therefore, the HP-DMEM gel with exogenously added cytokines provides an excellent 3D matrix for enhancing the proliferation of these cell types. Furthermore, when DFCs and MVECs were cultured in our 3D system using the HP-DMEM gel with F/P MPs and

Fig. 6 Proliferation of TF-1 cells in 2D and 3D cultures. Left panels: TF-1 cells (*upper figure*) were cultured in a 3D system using the HP-DMEM gel with F/P MPs and IL-3/GM-CSF (*filled square*), with F/P MPs alone (*open triangle*), IL-3/GM-CSF alone (*open square*) and no supplementation (*filled circle*). *Right panels* TF-1 cells (*upper figure*) were cultured in a 2D system with the HS-DMEM on F/P MP-coated plates (*open triangle*) with IL-3/GM-CSF (*filled square*), on uncoated plates (*filled circle*), with IL-3/GM-CSF alone (*open square*), and with

high concentration HS (10 %)-DMEM on uncoated plates (*open circle*). Proliferation of TF-1 cells was measured for 3 days. Data represent the mean $\pm$ SD for six measurements. *Lower panels* show photomicrographs of 3D and 2D cultures of TF-1 cells after 3 days of culture to illustrate their morphologies. The differences between the experimental groups in 3D culture (HP-DMEM gel with F/P MPs and IL-3/GM-CSF) and in 2D culture (HS-DMEM on F/P MP-coated plates with IL-3/GM-CSF) were significant (** $P < 0.01$ ($n = 6$))

FGF-2, we observed 3D granulation- and capillary-like tissue formation, respectively (data not shown).

In conclusion, we have established a 3D culture method for proliferation of DFCs, MVECs, SMCs and TF-1 cells using the HP-DMEM gel with F/P MPs and adequate cytokines such as FGF-2 or IL-3/GM-CSF. These results suggest that cells cultured by this method may be a promising tool for cell-based therapies in

many clinical fields, particularly in the preparation of a large number of various human cells.

Acknowledgments This study was partially supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan under Grant No. 23300181. Dr. Satoko Kishimoto was partially supported by Restart Postdoctoral (RPD) Fellowship of the Japan Society for the Promotion of Science.

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