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The effect of RGD peptide-conjugated magnetite cationic liposomes on cell growth and cell sheet harvesting

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Abstract

Tissue engineering requires novel technologies for establishing 3D constructs, and the layered method of culturing cell sheets (cell sheet engineering) is one potentially useful approach. In the present study, we investigated whether coating the culture surface with RGD (Arg–Gly–Asp) peptide-conjugated magnetite cationic liposomes (RGD-MCLs) was able to facilitate cell growth, cell sheet construction and cell sheet harvest using magnetic force without enzymatic treatment. To promote cell attachment, an RGD-motif-containing peptide was coupled to the phospholipid of our original magnetite cationic liposomes (MCLs). The RGD-MCLs were added to a commercially available 24-well ultra-low-attachment plate the surface of which comprised a covalently bound hydrogel layer that was hydrophilic and neutrally charged. A magnet was placed on the underside of the well in order to attract the RGD-MCLs to the surface of the well, and then NIH/3T3 cells were seeded into the well. Cells adhered to the bottom of the culture surface, which was coated with RGD-MCLs, and the cells spread and proliferated to confluency. After incubation, the magnet was removed and the cells were detached from the bottom of the plates, forming a contiguous cell sheet. Because the sheets contained magnetite nanoparticles, they could be harvested using a magnet inserted into the well. These results suggest that this novel methodology using RGD-MCLs and magnetic force, which we have termed 'magnetic force-based tissue engineering (Mag-TE)', is a promising approach for tissue engineering.

Keywords: Magnetite nanoparticles; Liposomes; RGD peptide; Tissue engineering; Cell culture

1. Introduction

Tissue engineering is a promising technology for overcoming the organ transplantation crisis resulting from donor shortage. Currently, tissue engineering is based on seeding cells onto three-dimensional (3D) biodegradable scaffolds, which allow the cells to reform their original structure [1]. However, some problems remain with this approach, for example, insufficient cell migration into the scaffolds and inflammatory reactions due to scaffold biodegradation, and thus novel approaches for achieving 3D tissue-like constructs are desired. A major difficulty obstructing the fabrication of in vivo-like 3D constructs without the use of artificial 3D scaffolds is a lack of cell adherence in the vertical direction via cell–cell junctions. This non-adherence may be caused by enzymatic digestion of adhesive proteins. To overcome this, Okano and colleagues employed a thermo-responsive culture surface grafted to poly(*N*-isopropylacrylamide) (PIPAAm) [2–4]. Cells adhered to and proliferated on the thermo-responsive surface, as well as on tissue culture polystyrene dishes. Furthermore, confluent cells on the PIPAAm dishes were expelled as intact contiguous sheets by decreasing the temperature to below the lower critical solution temperature (LCST) of PIPAAm [5]. Kushida et al. recovered monolayer cell sheets from a surface grafted with PIPAAm and then deposited extracellular matrices

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(ECMs); digestive enzymes were not used and ECMs remained to enhance cell–cell attachment [5]. These engineered cell sheets could be layered to construct multi-layered 3D cell sheets, and Okano et al. proposed the concept of 'cell sheet engineering' [4]. In the present study, we assessed a novel cell sheet engineering methodology using magnetic force and magnetite nanoparticles.

Magnetite nanoparticles are being used in an increasing number of biological and medical applications, including cell sorting [6–9]. We previously developed magnetite cationic liposomes (MCLs), which are cationic liposomes containing 10-nm magnetite nanoparticles, in order to improve accumulation of magnetite nanoparticles in target cells by taking advantage of the electrostatic interaction between MCLs (positively charged) and the cell membrane (negatively charged) [10–12]. In addition, we recently developed a tissue engineering technique using MCLs [13–15]. Our technique using MCLs consisted of the following processes: (1) MCLs were added to the target cells, which were expanded to the required cell number before applying to Mag-TE in order to magnetically label the cells through uptake of magnetite nanoparticles; (2) magnetically labeled cells were harvested and reseeded onto an ultra-low-attachment plate, the surface of which comprised a covalently bound hydrogel layer that was hydrophilic and neutrally charged, and a magnet was set at the underside of the well in order to attract and accumulate the cells, allowing the formation of 3D cell constructs; (3) the magnet was removed in order to detach the 3D cell constructs from the well; and (4) the 3D cell constructs were harvested using a magnet. We reported that magnetically labeled keratinocytes, which we accumulated using a magnet, resulted in a multi-layered 3D construct of keratinocytes [15]. Thus, we developed a novel methodology for tissue engineering using magnetite nanoparticles and magnetic force, which we designated 'Mag-TE'.

In the present study, we developed a new biomaterial and methodology for Mag-TE. The RGD (Arg–Gly–Asp) sequence, an integrin recognition motif found in fibronectin [16,17] and one of the most extensively studied cell adhesion peptides, was conjugated with magnetite cationic liposomes (RGD-MCLs). Here, we investigate the process of Mag-TE using RGD-MCLs.

2. Materials and methods

2.1. Cells and culture

Mouse NIH/3T3 fibroblasts were obtained from American Tissue Culture Collection. Cells were cultured at 37 °C under a humidified atmosphere of 5% CO_2 and 95% air in minimum essential medium (MEM) supplemented with 10% fetal calf serum, 10 mM non-essential amino acids, 0.1 mg/ml streptomycin sulfate and 100 U/ml potassium penicillin G.

2.2. Preparation of MCLs and RGD-MCLs

The magnetite (Fe_3O_4; average particle size, 10 nm) used as the core of the MCLs and RGD-MCLs was kindly donated by Toda Kogyo (Hiroshima, Japan). MCLs were prepared from colloidal magnetite and a lipid mixture consisting of *N*-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride (TMAG), dilauroylphosphatidyl-choline (DLPC), and dioleoylphosphatidylethanolamine (DOPE) in a 1:2:2 molar ratio, as described previously [10]. For RGD-MCLs, a lipid mixture consisting of TMAG, DLPC, and dioleoylphosphatidylethanolamine-*N*-[3-(2-pyridyldithio)-propionate] (PDP-DOPE, Sigma Chemical Co., St. Louis, MO) in a 1:2:2 molar ratio dissolved in chloroform was dried down by evaporation for a minimum of 30 min. Lipids were hydrated by vortexing in colloidal magnetite nanoparticles and the liposomes were sonicated for 30 min (28 W). Covalent coupling of RGDC (Arg–Gly–Asp–Cys) peptides with liposomes was carried out according to the method of Gyongyossy-Issa et al. [18]. Briefly, 2 ml of liposomes was mixed with RGDC peptides (Peninsula Laboratories Inc., Belmont, CA) to give an approximate molar ratio of 0.55 peptide to 1 PDP-DOPE which, in the case of liposomes, translates to a molar ratio of 1.1 peptides to 1 outside surface of PDP-DOPE. Coupling was carried out using gentle agitation at room temperature for 3.5 h in 2-morpholinoethanesulfonic acid (MES) buffer (pH 5.0). Peptide and magnetite concentrations were measured using the ninhydrin analysis method [19] and the potassium thiocyanate method [20], respectively. The size of RGD-MCLs was measured using a dynamic light scattering spectrophotometer (FRAR 1000, Otsuka Electronics, Osaka, Japan).

2.3. Cell culture on surface coated with RGD-MCLs

Media containing RGD-MCLs or MCLs at the indicated concentrations were added to a 24-well ultra-low-attachment plate (Corning, NY), the surface of which comprised a covalently bound hydrogel layer that was hydrophilic and neutrally charged. A cylindrical neodymium magnet (diameter, 30 mm; height, 15 mm; magnetic induction, 4000 G) was then placed on the underside of the low-attachment plate in order to provide magnetic force vertical to the plate, thus inducing the surface of the well to be coated with RGD-MCLs or MCLs. Approximately 2×10^4 NIH/3T3 cells were then seeded into the wells, and were cultured for the indicated periods. Viable cell number was measured using the dye-exclusion method with

trypan blue. Levels of statistical significance in the number of viable cells were evaluated using the Mann–Whitney rank sum test [21].

2.4. Cell sheet construction using RGD-MCLs and magnetic force

After 4 d of incubation on RGD-MCLs, the magnet placed on the underside of the 24-well ultra-low-attachment plate was removed. Subsequently, the medium was gently pipetted in order to induce medium flow from the periphery of the cell sheets.

For histological evaluation of cell sheets, cells were washed with phosphate-buffered saline (PBS), fixed in 10% formalin solution and embedded in paraffin. Thin (4 μm) slices were stained with hematoxylin and eosin [15]. Immunohistochemistry for fibronectin using mouse anti-fibronectin antibody was also performed in order to assess ECM deposition. Sections were incubated with 10% skim milk at 37 °C for 10 min to block background staining. They were then incubated at 37 °C for 60 min with mouse monoclonal antibody preparations (MAbs) for fibronectin (clone: TV-1, Chemicon, CA) antigens, and then at 37 °C for 60 min with biotinylated goat anti-mouse IgG. Slices were then incubated at 37 °C for 30 min with peroxidase-conjugated streptavidin (DAKO. Japan, Kyoto). Each step was followed by washing with phosphate buffer. Peroxidase activity was visualized by treatment at room temperature for 10 min with 0.02% diaminobenzidine tetrahydrochloride solution containing 0.005% hydrogen peroxide. As a negative control, primary antibodies were replaced with unrelated monoclonal antibodies.

2.5. Recovery of NIH/3T3 cell sheets using magnets

When the magnet placed on the underside of the 24-well ultra-low-attachment plate was removed, the cell sheets constructed by Mag-TE were harvested using a cylindrical alnico magnet (diameter, 10 mm; height, 50 mm; magnetic induction, 1200 G) by approaching from the top of the cell sheet. A hydrophilically treated poly(vinylidine difluoride) (PVDF) membrane (Amersham, Buckinghamshire, England) was placed on the head of the alnico magnet to prevent direct attachment of cell sheets to the magnet. The magnet was positioned at the surface of the culture medium to ensure that the cell sheets were attracted by the magnetic force and the cell sheets became bound to the PVDF membrane.

In order to determine the magnetite content of the sheets, iron concentration was measured using the potassium thiocyanate method, as described previously [15].

3. Results

3.1. Preparation of RGD-MCLs

RGD-MCLs, which contained magnetite nanoparticles that possessed a positively charged cationic lipid surface and were coupled with the cell adhesion peptide RGD, were constructed. Our original MCLs, which contained magnetite nanoparticles with a positively charged surface, were also prepared as a control. The size distribution of MCLs and RGD-MCLs is shown in Fig. 1. The average particle size of RGD-MCLs was 243 ± 63.2 nm, and this value was similar to that of MCLs (188 nm). The density of the immobilized peptides in RGD-MCLs was 0.226 mg peptide/mg magnetite.

3.2. Cell culture on the culture surface coated with RGD-MCLs

The optimum concentration of RGD-MCLs for cell culture was investigated (Fig. 2A). Various concentrations (0–30 μg/well) of RGD-MCLs or MCLs were added to the medium. When a cylindrical neodymium magnet (diameter, 30 mm; height, 15 mm) was placed on

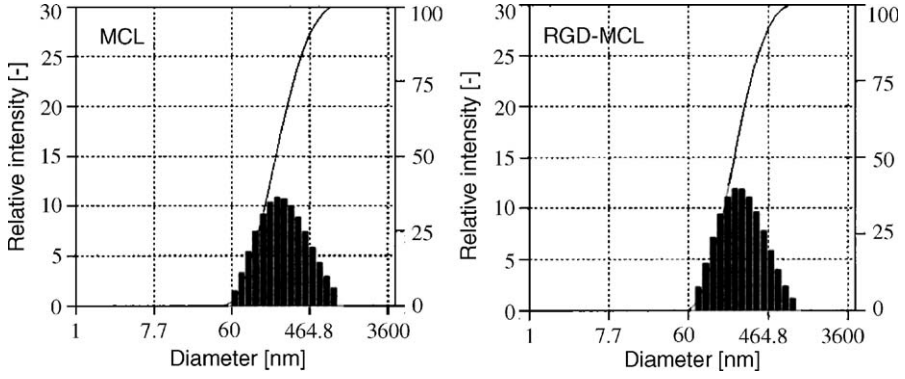


Fig. 1. Size distribution of MCLs and RGD-MCLs. Representative DLS data are shown.

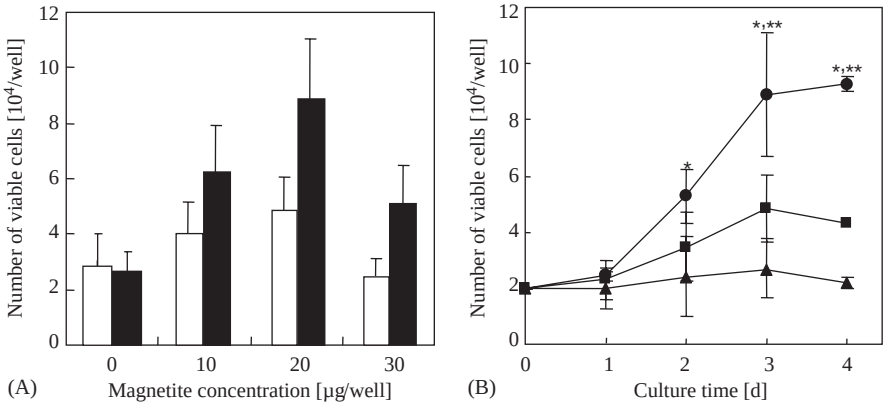


Fig. 2. Cell proliferation of NIH/3T3 cells on the surface coated with RGD-MCLs. Media containing MCLs or RGD-MCLs were added to a 24-well ultra-low-attachment plate, and a cylindrical neodymium magnet was placed on the underside of the low-attachment plate. Approximately 2×10^4 NIH/3T3 cells were then seeded into the wells. (A) The optimum magnetite concentration of MCLs (open symbols) or RGD-MCLs (closed symbols) was investigated by varying the concentration from 0 to 30 μg/well. The number of viable cells after a 3-d incubation period was measured using the dye-exclusion method with trypan blue. Data and bars represent mean ± SD of 3 independent experiments. (B) Growth curve of cells cultured on an uncoated surface (triangles), MCLs (20 μg/well, squares), and RGD-MCLs (20 μg/well, circles). On the indicated day, the number of viable cells was measured by the trypan blue exclusion method using a hemocytometer. Data points represent the mean ± SD of 3 independent experiments. $^*p < 0.05$, non-coated surface vs RGD-MCLs; $^{**}p < 0.05$, MCLs vs RGD-MCLs.

the underside of the 24-well ultra-low-attachment plate to provide magnetic force vertical to the plate, RGD-MCLs or MCLs in the medium were attracted to the culture surface. With the magnet remaining on the underside, 2×10^4 NIH/3T3 cells were seeded into the wells and cultured for 4 d. Fig. 2A shows the number of viable cells after the 4-d culture period. For RGD-MCLs, the highest number of viable cells (8.9×10^4) was observed at a concentration of 20 μg/well. For MCLs, the optimum magnetite concentration was also found to be 20 μg/well. Consequently, in all subsequent experiments, RGD-MCLs or MCLs were added to the media at a concentration of 20 μg/well.

The growth curve of cells cultured on RGD-MCLs is shown in Fig. 2B. When NIH/3T3 cells, which are anchorage dependent, were cultured in 24-well ultra-low-attachment plates, the cell number did not change during the 4-d incubation period. When the culture surface was coated with MCLs, the number of viable cells increased slightly and was two-fold higher after the 4-d incubation period. For RGD-MCLs, an apparent cell proliferation was observed and the cells proliferated to confluency during the 4-d incubation period.

Fig. 3 shows photomicrographs of NIH/3T3 cells cultured on the uncoated surface (I), the surface coated with MCLs (II), and that coated with RGD-MCLs (III) for 1 d (A) or 4 d (B). When the cells were cultured in 24-well ultra-low-attachment plates, the cells floated in the media (Fig. 3B) and no cells were observed to be attached to the plate (data not shown). Subsequently, the cells aggregated and formed spheroid constructs on day 4. For MCLs, although cells were observed to be attached to the culture surface, very few spreading cells were seen (Fig. 3A). On the other hand, when the cells were cultured on the surface coated with RGD-MCLs, NIH/3T3 cells had adhered and spread on day 1 (Fig. $3A_{III}$), and had proliferated to confluency on day 4 (Fig. $3B_{III}$).

3.3. Construction and harvest of cell sheets

The NIH/3T3 cells grown to confluency on RGD-MCLs were subjected to a novel fabricating and harvesting method using a magnet. Fig. 4 shows bright-field photographs of cells cultured on RGD-MCLs for 4 d. The cell sheets containing magnetite nanoparticles had a black–brown color. After 4 d of incubation on RGD-MCLs, the magnet placed on the underside of the 24-well ultra-low-attachment plate was removed. When the medium was gently pipetted to cause medium flow from the periphery of cell sheets, cells were detached from the bottom of the plates and shrunk, thus resulting in the formation of a contiguous cell sheet-like construct (Fig. 4A). To confirm that NIH/3T3 cells formed a sheet-like construct, we examined cross-sections of obtained cell sheets. Phase-contrast microscopy of the cross-sections revealed that one to two cell layers were present throughout the cell sheets in the 24-well tissue culture plates (Fig. 4B). Immunohistochemical staining revealed that fibronectin, which is the major ECM component to mediate cell adhesion, was deposited in the cell sheets (Fig. 4C).

The cell sheets fabricated by Mag-TE had a black–brown color due to magnetite nanoparticles, and we investigated whether these cell sheets could be harvested using magnets. The sheets cultured using RGD-MCLs

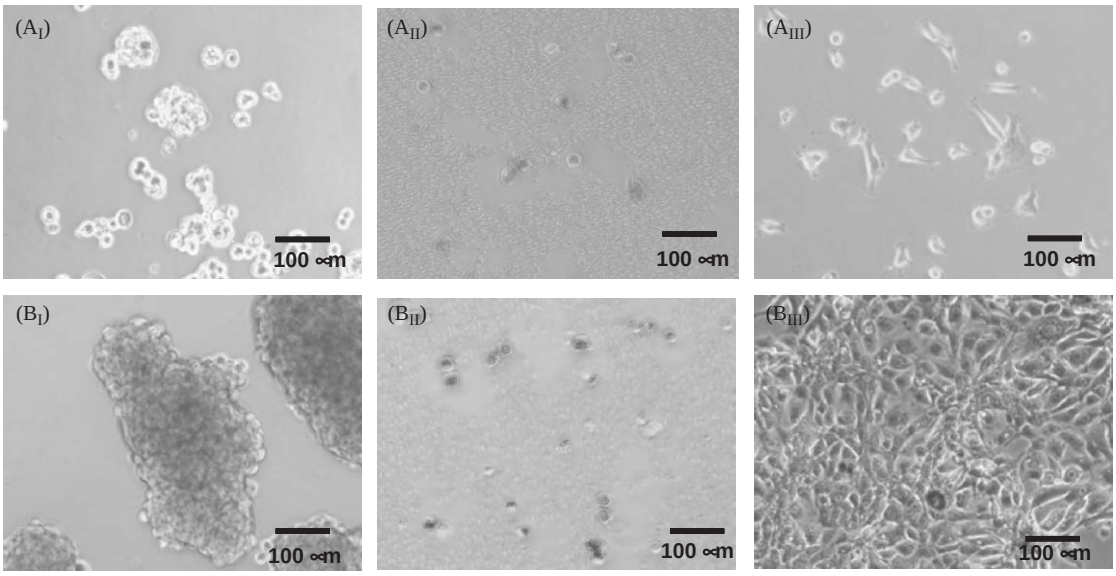


Fig. 3. Photomicrographs of NIH/3T3 cells cultured on an uncoated (I), a surface coated with MCLs (II), and that coated with RGD-MCLs (III) for 1 d (A) or 4 d (B). With the uncoated surface (A_I and B_I), cells floated, while with MCLs (A_{II} and B_{II}) and RGD-MCLs (A_{III} and B_{III}), cells became attached to the culture surface.

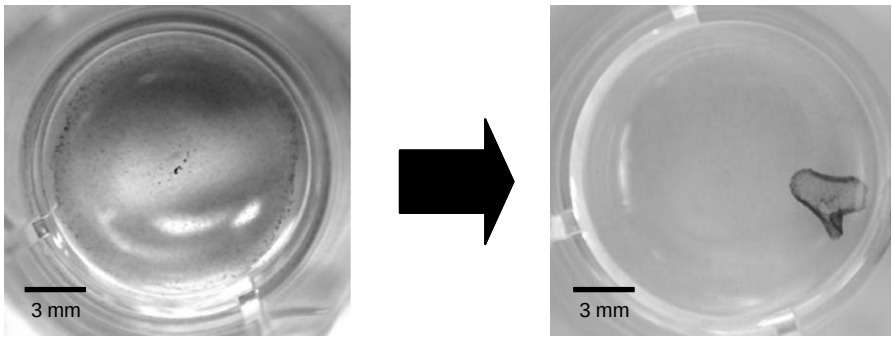
contained 8.31 μg magnetite nanoparticles per sheet. Fig. 5 shows the actual procedure for the harvest of cell sheets constructed by Mag-TE. The cylindrical neodymium magnet positioned on the underside of the 24-well ultra-low-attachment plate was removed. A hydrophilically treated PVDF membrane was the pasted to the head of a cylindrical alnico magnet (Fig. 5A), and the magnet moved into the well toward the surface of the culture medium (Fig. 5B). Due to the magnetic force, the cell sheets floated up to the surface of the culture medium and without disruption, stuck to the PVDF membrane, as shown in Fig. 5C.

4. Discussion

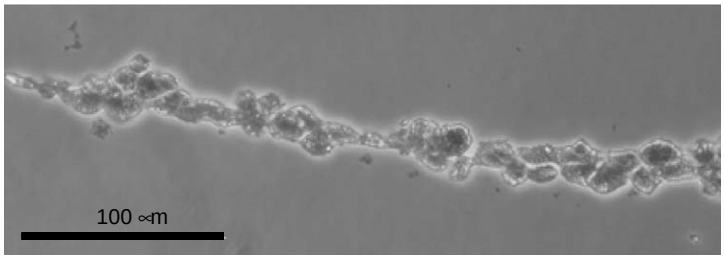
4.1. Effects of RGD-MCLs on cell adherence

In the present study, cell integrin ligand RGD peptides were covalently coupled to cationic liposomes containing magnetite nanoparticles, and we used these RGD-MCLs as a novel biomaterial for tissue engineering. RGD-MCLs have two possible active effects on cell adherence; one is positive charge of the cationic liposomes, and the other is the RGD peptide on their surface. When NIH/3T3 cells were cultured in 24-well ultra-low-attachment plates, the cells floated in the media and formed spheroid constructs (Fig. 3). When cells were cultured on the surface of neutrally charged magnetoliposomes [10], the cells floated in the media and no cells were observed to be attached to the plate (data not shown). On the other hand, cells attached onto the culture surface coated with MCLs, thus suggesting that positive charge due to cationic liposomes facilitated cell attachment to the culture surface. However, few NIH/3T3 cells spread and proliferated on MCLs. In contrast, when cells were cultured on the surface coated with RGD-MCLs, the cells adhered, spread, and proliferated (Fig. 3), thus suggesting that RGD peptides strongly promoted cell adhesion. The mechanism by which RGD-MCLs facilitate cell adhesion and proliferation has not been fully elucidated. In our preliminary study, transmission electron microscopy (TEM) revealed that approximately 50% of totally MCLs in the field of vision bound to cell surfaces while the remainder was situated in the cytoplasm when MCLs were added to cells (data not shown). Although the endocytotic behavior of RGD-MCLs has not been investigated, we speculated that some RGD-MCLs were endocytosed while RGD-MCLs that were bound to the cell surface facilitated cell adhesion and proliferation. Further study regarding morphological changes when RGD-MCLs are incubated with cells may clarify the mechanism of 'cell to RGD-MCL' interactions.

Although several researchers have reported that cell adhesion due to RGD peptides depends on the concentration of immobilized RGD peptides [22–24], higher concentrations of RGD-MCLs (30 μg/well) did not result in optimum conditions for cell adhesion. This may be due to the toxicity of cationic liposomes and/or magnetite nanoparticles, because higher concentrations of MCLs (30 μg/well) did not result in optimum



(A)



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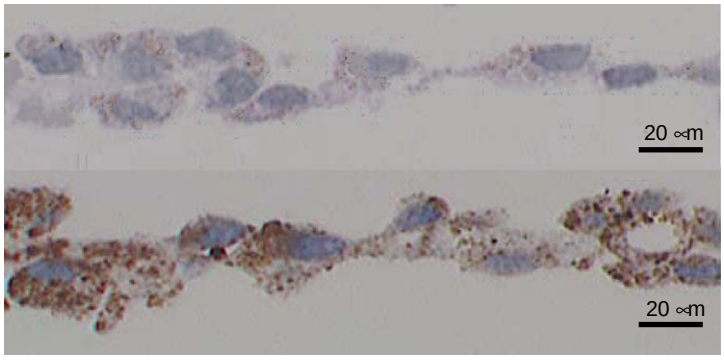

(C)

Fig. 4. Cell sheets fabricated by Mag-TE using RGD-MCLs. (A) Bright-field photographs of confluent cells together with a magnet (left) and cell sheets after removal of the magnet (right). (B) Hematoxylin and eosin-stained cross-sections of NIH/3T3 sheets constructed using RGD-MCLs and a magnet. (C) Immunohistochemical staining with anti-fibronectin antibody (bottom) and isotype control antibody (IgG_1) staining (top).

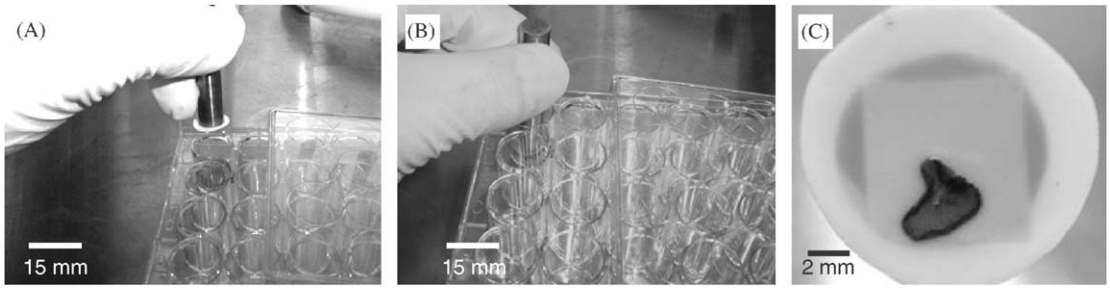

Fig. 5. Recovery of NIH/3T3 cell sheets using magnets. (A) In order to harvest the NIH/3T3 cell sheets containing RGD-MCLs, the magnet positioned on the underside of the 24-well ultra-low-attachment plate was removed, and a hydrophilically treated PVDF membrane was placed on top of a cylindrical alnico magnet. (B) The magnet was positioned at the surface of culture medium. (C) Due to the magnetic force, the NIH/3T3 sheets floated up to the surface of the culture medium and stuck to the PVDF membrane.

conditions for cell adhesion either. These results suggest that cell proliferation was enhanced when the immobilization density of RGD peptide on MCLs was increased. Here, toxic effects of MCLs on cell proliferation or differentiation may differ between cell types; we have examined several types of cells, including human keratinocytes (MCL administration at 1 μg magnetite/well) [15], human aortic endothelial cells (2 μg/well) [14], and human mesenchymal stem cells (MSCs, 2 μg/well) [13], and no toxicity was observed when MCLs were administrated below the indicated concentrations. In the present study, although RGD-MCLs or MCLs were used at the higher concentrations of 10 to 30 μg/well, proliferation of NIH/3T3 cells were strongly promoted at 20 μg/well (Fig. 2B) and dying cells were rarely observed at 30 μg/well (data not shown), which indicates that RGD-MCLs represent a potent biomaterial for tissue engineering. These data encourage further development of tissue engineering techniques using magnetite cationic liposomes coupled with cell adhesion peptides, such as KQAGDV (Lys–Gln–Ala–Gly–Asp–Val) peptide for smooth muscle cells [25] and YIGSR (Tyr–Ile–Gly–Ser–Arg) peptide for neurons [26].

4.2. Fabrication of cell sheets

We previously developed a tissue engineering technique using MCLs. On the other hand, RGD-MCLs developed in the present study facilitated 'cell growth', which was hardly observed when MCLs were used (Fig. 2B), even though initial adhesion of the cells did not change between RGD-MCLs and MCLs (Fig. 3). This new function enabled cell growth and cell sheet construction to be performed simultaneously, while these two processes are clearly separated in Mag-TE using MCLs: First, cells were cultured to obtain sufficient numbers in tissue culture polystyrene dishes; then, magnetically labeled cells were accumulated using a magnet, resulting in cell sheet formation. Thus, we have demonstrated an alternative methodology for rapid cell sheet construction using RGD-MCLs.

Fabrication of 3D constructs without using 3D scaffolds, including 'cell sheet engineering' as proposed by Okano et al. [2–5,23], may be an alternative approach to tissue engineering. When cells are cultured to confluency, they connect with one another via ECMs. Upon enzymatic digestion, these proteins are disrupted and the cells are released separately. In the case of cell sheet engineering using a PIPAAm-grafted surface, cell-to-cell connections are not disrupted and cells are harvested as a contiguous cell sheet by decreasing temperature. Furthermore, cell adhesion proteins in cell sheets are also maintained and they play a role as adhesive agents in transferring cell sheets onto other cell sheets. In the present study, we developed a novel methodology for fabrication of cell sheets without enzymatic treatment, and we have designated this technique 'Mag-TE'. When NIH/3T3 cells were seeded onto RGD-MCLs, they adhered, spread, and proliferated to confluency (Fig. $3B_{III}$). The confluent cells could be harvested as contiguous cell sheets, as shown in Fig. 4A, after removing the magnet placed on the underside of the plates. Pathological analysis using immunohistochemical staining revealed that these contiguous cell sheets contained substantial amounts of fibronectin proteins (Fig. 4C), thus suggesting that RGD-MCLs mediated ECM deposition and cell adhesion onto ECMs. These results demonstrate that Mag-TE using RGD-MCLs allowed cell harvest using magnetic force without enzymatic digestion. However, harvested cell sheets shrank considerably, as shown in Fig. 4A, but this contraction may be prevented by using supports, such as PVDF membranes [4] or possibly other magnetic devices.

4.3. Harvest of cell sheets by magnetic force

In cell sheet engineering, methodologies for handling cell sheets are needed because grafts fabricated by cell sheet engineering are easily damaged to handling. We proposed novel methodology for handling cell sheets using RGD-MCLs and magnetic force; the Mag-TE method enabled us to handle cell sheet-engineered grafts. Because cell sheets constructed by Mag-TE contained magnetite nanoparticles, NIH/3T3 cell sheets could be recovered and handled using a magnet (Fig. 5). This feature is not present in cell sheets produced using other methodologies, such as the use of a PIPAAm-grafted surface. These results also suggest the possibility of developing a 'tissue-engineered graft delivery system' using Mag-TE, while Mag-TE could also be applied to industrial tissue engineering because magnetic force could greatly simplify the recovery step of cell sheet production. As shown in Fig. 5, due to the magnetic force, the cell sheets floated up to the surface of the culture medium and stuck to the PVDF membrane. This recovery step could be automated by substituting the magnet used to harvest cell sheets with an electromagnet, which can immediately release the sheets from the surface of the magnet via electrical control. These findings indicate that RGD-MCLs are potent tools for industrial tissue engineering.

4.4. Toxicity of RGD-MCLs

Information on the toxicity of RGD-MCLs is important for clinical applications. As mentioned above, MCLs are non-toxic against proliferation of several cell types and no effects on the differentiation of MSCs were noted when the magnetite concentration was below 2 μg/well. Moreover, in our preliminary study, the toxicity of systemically administered of MCLs (90 mg, i.p.) in mice

was investigated, and none of the ten mice died after MCL administration. Transient accumulation of magnetite was observed in mouse liver and spleen, but the particles were cleared from circulation by hepatic Kupffer cells and/or fixed macrophages in the spleen by the 10th day after administration [27]. In the present study, the NIH/3T3 cell sheets contained only 8.31 μg of MCLs, which is far less than the toxic level of MCLs (90 mg). However, further study regarding the toxicity of residual magnetic nanoparticles in the grafts will be needed before any clinical application. Cytotoxicity assay of magnetic nanoparticles having different properties, including various particle sizes, may also be needed in preclinical in vivo experiments.

5. Conclusions

We developed a novel methodology to construct cell sheets using RGD-MCLs and magnetic force, which we designated 'Mag-TE'. Mag-TE allowed us to fabricate and harvest cell sheets, and may be applied to tissue engineering of 3D tissues.

Acknowledgements

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