Autologous extracellular matrix scaffolds for tissue engineering

Hongxu Lu a,b, Takashi Hoshiba a, Naoki Kawazoe a,c, Guoping Chen a,b,c,*

a Biomaterials Center, National Institute for Materials Science, 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan
b Graduate School of Pure and Applied Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8577, Japan
c International Center for Materials Nanoarchitectonics (MANA), National Institute for Materials Science, 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan

1. Introduction

Tissue engineering and regenerative medicine have been rapidly developed as an encouraging alternative practice to restore or replace lost or malfunctioning tissues and organs through the use of cells and biomaterial scaffolds [1–3]. In a common approach, the cells isolated from a patient are cultured in a biocompatible three-dimensional (3D) porous scaffold supplemented with growth factors to regenerate new tissues or organs [4,5]. The scaffold provides necessary intermix support for cell adherence, proliferation, and phenotypic differentiation; offers biochemical and biophysical cues to modulate the neo-tissue formation by mimicking the functional and structural characteristics of the native ECM [6,7], which play a crucial role in controlling and regulating cell behavior and function [8,9].

Thus far, scaffolds have been developed from synthetic biodegradable polymers such as poly(lactic acid) (PLA), polylactide-co-glycolide (PLGA) [10]; natural polymers such as collagen, chondroitin, and hyaluronic acid [10,11]; and acellular matrices derived from decellularized tissues and organs [12–15]. However, the synthetic polymers are limited by their biological inertness [16] and the acidic moieties, residual catalysts, and microscale particulates that accompany degradation [17]. On the other hand, although the naturally derived polymer and acellular matrices can provide abundant biological signals and degrade into physiologically tolerable compounds [18], they are exhaustively xenogeneic or allogeneic. This situation adds potential risks of pathogen transmission [19,20], and provocation of undesirable inflammatory and immunological reaction, leading to unfulfilled results from the regenerated tissues and organs [21–26]. To avoid these problems, the autologous extracellular matrix (aECM) scaffold should be a safe and reliable biomaterial candidate [27]. The development of aECM scaffolds has been strongly anticipated for use in tissue engineering and regenerative medicine [28,29]. The use of both autologous cells and autologous scaffolds would eliminate negative host responses and lead to optimal tissue regeneration. However, the availability of autologous sources of donor tissues and organs is highly limited. It has been almost impossible to use such acellular autologous matrices for tissue engineering. The ECM secreted by autologous cells would be a potential alternate to acellular autologous tissues and organs because some autologous cells can be expanded in vitro and maintained under a pathogen-free condition.

In this study, we developed a method of preparing ECM scaffolds by the 3D culture of cells in a selectively removable template (Fig. 1). The intracellular components and the biodegradable polymer template were selectively removed after cell culture to obtain the ECM scaffolds. To confirm the effectiveness of this method, three cell types, human bone marrow mesenchymal stem cells (MSCs), normal human articular chondrocytes (NHAC), and normal human dermal fibroblasts (NHDF) were used to prepare their respective ECM scaffolds. If autologous cells were used, the method...
could be used to prepare aECM scaffolds. To demonstrate the biocompatibility of the aECM scaffolds, we implanted mouse fibroblast-derived ECM scaffolds (ECM-mF) into ICR (C57:CD1) mice to evaluate the host tissue responses.

2. Materials and methods

2.1. ECM scaffold fabrication and characterization

MSC and NHAC, both at passage 2, were obtained from Lonza (Walkersville, MD). NHDF (derived from neonatal foreskin) were purchased from Cascade Biologics (Invitrogen, Portland, OR). The cells were seeded in 75 cm² tissue culture flasks and cultured using their respective proliferation media under an atmosphere of 5% CO₂ at 37 °C. MSC were cultured in MSC basal medium with MSC growth supplement; NHAC were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum; NHDF were cultured in Medium 106 with low serum growth supplement. The details were listed in Supplementary Tables 1 and 2. The cells were further subcultured thrice after confluence. Cells at passage 5 were collected by treatment with trypsin/EDTA solution and re-suspended in the medium for scaffold fabrication.

A knitted PLGA mesh (Vicryl polyglactin 910, Johnson & Johnson, Somerville, NJ) was used as a template to fabricate the ECM scaffolds. The mesh was cut into small discs with a diameter of 10.4 mm. Cells at passage 5 were seeded in discs of knitted PLGA mesh to form cell-PLGA constructs. The cell densities of MSC, NHAC, and NHDF were 3 × 10⁶, 5 × 10⁵, and 5 × 10⁴ cells/ml, respectively; 200 µl cell suspension was seeded on one side of each PLGA disc. Glass rings (inner Ø = 10 mm, outer Ø = 12 mm) were covered on PLGA discs to prevent cell leakage during cell seeding. After culture for 6 h, the cell-seeded PLGA mesh discs were turned over and the other sides of the mesh discs were also seeded with the same number of cells. The cell-ECM-PLGA constructs were formed by culturing cell-PLGA constructs in DMEM supplemented with 10% fetal bovine serum; NHDF were cultured in Medium 106 with low serum growth supplement. The constructs were obtained after decellularization, the ECM scaffolds were stained with Hoechst 33258 (for cell nuclei), Alexa Fluor 488-labeled phalloidin (for F-actin), and DiI (for cell membrane). The cell-ECM constructs without decellularization and PLGA were also stained as controls. The fluorescent staining was observed under an Olympus BX51 fluorescence microscope with a DP-70 CCD camera (Olympus, Tokyo, Japan). The images were manipulated by DP controller software.

Immunofluorescence staining was performed to confirm the existence of ECM components in the scaffolds, including type I collagen, type II collagen, type III collagen, fibronectin, vitronectin, laminin, aggregan, versican, decorin, and biglycan. The antibodies are listed in Supplementary Table 3. Briefly, the ECM scaffolds were incubated with 2% BSA/PBS for 30 min for blocking. The ECM scaffolds were incubated with 0.1M phosphate buffer (pH 6.0), with 5 mM cysteine hydrochloride, and 5 mM EDTA. The lysates were used for detection of the DNA and GAG amount. A Hoechst 33258-based DNA quantification kit (Sigma) was used to measure the DNA. Five µl lysate was added to 2 ml Hoechst 33258 solution and the fluorescence intensity was recorded by a FP-6500 spectrofluorometer (Jasco, Tokyo, Japan). The excitation wavelength was set at 360 nm and the emission wavelength was set at 460 nm. The DNA amount was calculated based on a standard curve obtained with the standard DNA supplied with the kit (n = 3). The sulfated glycosaminoglycan (GAG) amounts were measured by a Blyscan™ GAG assay kit (Biocolor, Newtonabbey, UK) according to the manufacturer’s manual. The specimen lysate was mixed with Blyscan™ dye to bind the GAG. The GAG-dye complex was then collected by centrifugation. After the supernatant was removed and the tube drained, the dissociation reagent was added. Then 200 µl solution was transferred into a 96-well plate. Absorbance against the background control was obtained at a wavelength of 656 nm on a Benchmark Plus™ microplate spectrophotometer (Bio-Rad, Tokyo, Japan) and the GAG amount (n = 3) was calculated based on a standard curve obtained with the standard GAG supplied with the kit.

Collagen contents in the ECM scaffolds were determined using the Sircol collagen assay (Biocolor) according to the manufacturer’s instructions. In brief, ECM scaffolds were incubated for 48 h at 48 °C in 0.5 N acetic acid containing 0.1 mg/ml pepsin. The samples were added to Sircol dye reagent, and collagen-dye complexes formed and precipitated out from the soluble unbound dye. After centrifugation, the pellet was washed once with Acid-Salt Wash Reagent and suspended in alkaline.
Fig. 2. SEM images of PLGA mesh (a), MSC-ECM-PLGA constructs (b), NHAC-ECM-PLGA constructs (c) and NHDF-ECM-PLGA constructs (d). MSC were cultured in the PLGA disc for 5 days; NHAC and NHDF were cultured for 6 days. Scale bar, 200 μm.

Fig. 3. Gross appearance of a mesh-like ECM-M (a), ECM-C (b), and ECM-F (c) and SEM images of the ECM scaffolds (d–i). (g–i), High magnification of (d), (e), and (f), respectively. Scale bar = 500 μm in (d–f) and 50 μm in (g–i).
reagent. The solution (200 µl) was transferred to a 96-well plate and the absorbance was read at 555 nm. The amount of collagen (n = 3) was calculated based on a standard curve obtained with the standard bovine type I collagen supplied with the kit.

2.3. Fabrication of ECM scaffolds from mouse fibroblasts and in vivo implantation

The animal experiment was conducted according to the committee guidelines of the National Institute for Materials Science of Japan for Animal Experiments. Surgical plane anesthesia in each animal was induced and maintained with 2% isoflurane in oxygen. Mouse fibroblasts (mF) were isolated from the biopsies of 6-week-old ICR (Crlj:CD1) mice and expanded in vitro according to the protocol described by A. Takashima [32]. P2 mouse fibroblasts were seeded (5 × 10⁵ cells/ml, 200 µl/side) and cultured in PGA meshes for 10 days. ECM-mF scaffolds were fabricated by culturing the mouse fibroblasts in the PGA mesh using the same method described above.

Thirty mice were randomly divided into 5 groups. The aECM groups were implanted with aECM derived from autologous cells. The Allo groups were implanted with ECM scaffolds derived from the allogeneic cells. After being steriley cut into Ø 8 mm discs, bovine collagen sponge (BCS) (Koken, Tokyo, Japan) and PLGA knitted mesh (Johnson & Johnson) were separately implanted into the mice. Mice that were opened but without implantation (sham operation) were used as controls. The central back was shaved and opened for implantation in a sterile fashion. The materials were subcutaneously implanted in the dorsum of each mouse. The openings were then sutured with nylon sutures.

2.4. Evaluation of host responses

One week after implantation, the mice were scarified. The back skin and the sample-harboring tissues were excised and fixed in 10% neutral buffered formalin solution. The specimens were trimmed and embedded in paraffin, and then sectioned at 7-µm intervals. The tissue sections were stained with hematoxylin and eosin. Because the ECM scaffolds were invisible after implantation (Supplementary Fig. 1), we excised large enough areas to ensure that the scaffolds were held in the specimens. During sectioning, each sample was labeled for several domains and representative slices of each domain to locate the implanted materials. The sections in positive domains were used for further exploration.

Immunocytochemical staining was performed to identify immunocytes by using anti-F4/80, anti-Gr-1, anti-CD3, anti-MHC II monoclonal antibodies with a Vectastain Elite ABC kit (Vector, Burlingame, CA). The detailed information of antibodies was listed in Supplementary Table 4. Briefly, the slides were deparaffinized and hydrated. Then a heat-mediated antigen retrieval technique that included

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**Fig. 4.** Fluorescence staining of F-actin, cell nuclei, and cell membrane for the confirmation of decellularization in ECM-M (a) and ATR-FTIR spectra of PLGA template, cell-ECM-PLGA construct, and ECM-M (b). Scale bars = 100 µm in (a).
2. Statistical analysis

2.5. Statistical analysis

Statistical analysis. All results are presented as the mean ± standard deviation (SD). A two-tailed t-test was used to examine the statistical differences of DNA, sGAG, and soluble collagen contents between decellularized and un-decellularized samples. A one-way analysis of variance (ANOVA) was used to analyze the statistical differences in host tissue response. If the overall ANOVA test was significant, a Tukey’s post hoc test was performed for a pairwise comparison. A P-value <0.05 was considered statistically significant.

3. Results and discussion

3.1. Fabrication and characterization of ECM scaffold

Bone marrow mesenchymal stem cells (MSCs), dermal fibroblasts, and articular chondrocytes, were used to prepare their respective ECM scaffolds. MSCs, fibroblasts, and chondrocytes were seeded and cultured in templates of PLGA knitted mesh (Fig. 2a), which were used as temporary skeletons. The cells adhered, proliferated, and secreted ECM in the templates. Fig. 2b–d show the distribution of cells and their secreted ECM in the PLGA mesh after culture for several days. After culture for 5 (MSC) or 6 (NHAC and NHDF) days, the cellular components were removed by the decellularization method of freeze-thaw cycling plus the treatment with ammonium hydroxide. The PLGA mesh templates were selectively removed by the treatment of immersion in 0.5 M Na3PO4 aqueous solution at 37 °C for 48 h. The freeze-thaw cycling causes cryoinjuries to cells through osmotic response, extracellular and intracellular ice formation [33]. After repeated freeze-thaw cycling, cells were broken and released intracellular components. After NH4OH is added, the connections between intracellular and extracellular molecules will be broken. Therefore, cellular debris could be removed after complete washing. The weak alkaline environment of 0.5 M Na3PO4 could also contribute to the removal of cellular components (such as DNA). The resulting ECM scaffolds prepared from MSC, NHAC, and NHDF were designated as ECM-M, ECM-C, and ECM-F, respectively.

The ECM scaffolds exhibited a mesh-like appearance similar to that of the temporary skeletal PLGA knitted mesh (Fig. 3a–c). The

Table 1
DNA contents (ng/mesh) in the cell-ECM-PLGA constructs and the ECM scaffolds.

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<tr>
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<th>Cell-ECM-PLGA constructs</th>
<th>ECM</th>
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<tbody>
<tr>
<td>ECM-M</td>
<td>670.11 ± 51.54*</td>
<td>4.38 ± 1.67</td>
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<tr>
<td>ECM-C</td>
<td>2677.89 ± 511.35*</td>
<td>2.79 ± 0.63</td>
</tr>
<tr>
<td>ECM-F</td>
<td>2698.33 ± 567.10*</td>
<td>4.35 ± 0.81</td>
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</table>

Data represent means ± S.D. (n = 3).

* Significant difference compared with ECM scaffold, P < 0.001.

Table 2
sGAG (ng/mesh) contents in the cell-ECM-PLGA constructs and the ECM scaffolds.

<table>
<thead>
<tr>
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<th>Cell-ECM-PLGA construct</th>
<th>ECM</th>
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<tbody>
<tr>
<td>ECM-M</td>
<td>916.85 ± 41.39*</td>
<td>54.72 ± 31.79</td>
</tr>
<tr>
<td>ECM-C</td>
<td>1937.41 ± 104.57*</td>
<td>61.21 ± 26.74</td>
</tr>
<tr>
<td>ECM-F</td>
<td>2436.67 ± 120.38*</td>
<td>51.93 ± 10.89</td>
</tr>
</tbody>
</table>

Data represent means ± S.D. (n = 3).

* Significant difference compared with ECM scaffold, P < 0.001.

Fig. 5. Immunofluorescence images of ECM molecules composing ECM-M. Scale bar, 100 μm.
porous 3D structure, microscale and nanoscale ECM fibers, was observed by a scanning electron microscope (SEM) (Fig. 3d–i). However, there was no obvious difference among ECM-M, ECM-C, and ECM-F in terms of morphology. The geometrical properties, porosity, interconnectivity, and nanoscaled fibrous structure are meant to support cell proliferation and differentiation, and benefit tissue regeneration [34,35].

The cell nuclei, cell membrane, and F-actin were removed by the decellularization treatment (Fig. 4a, and Supplementary Fig. 2a and b). Most of the DNA was removed and only a very small amount remained in the scaffolds. The amount of DNA in ECM-M, ECM-C, and ECM-F was 0.65%, 0.10%, and 0.16% of the values before decellularization, respectively (Table 1). ATR-FTIR spectra verified the complete removal of the PLGA templates from the ECM scaffolds because the typical band for the ester carbonyl stretch at 1740 cm$^{-1}$ disappeared in the ECM scaffolds but was present in the cell-ECM-PLGA complexes (Fig. 4b, and Supplementary Fig. 2c and d). The PLGA template was removed because its degradation might provoke adverse inflammation. Decellularization could facilitate the removal of the PLGA template and avoid undesired effects such as calcification induced by the cellular components of DNA and lipids [36].

The compositional biomolecules in ECM-M, ECM-C, and ECM-F were analyzed by immunohistological staining. There were some differences among the components of the ECM-M, ECM-C, and ECM-F (Supplementary Table 5). ECM-M consisted of type I collagen, type III collagen, fibronectin, vitronectin, laminin, aggrecan, decorin, and biglycan (Fig. 5). ECM-C consisted of type I collagen, type III collagen, fibronectin, vitronectin, laminin, aggrecan, versican, decorin, and biglycan (Supplementary Fig. 3a). ECM-F consisted of type I collagen, type III collagen, fibronectin, vitronectin, laminin, decorin, and biglycan (Supplementary Fig. 3b).

### Table 3

<table>
<thead>
<tr>
<th>Soluble collagen contents (µg/mesh) in the cell-ECM-template constructs and the ECM scaffolds.</th>
<th>Cell-ECM-PLGA construct</th>
<th>ECM</th>
</tr>
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<tbody>
<tr>
<td>ECM-M</td>
<td>5.83 ± 0.88$^a$</td>
<td>2.78 ± 0.34</td>
</tr>
<tr>
<td>ECM-C</td>
<td>4.34 ± 0.39$^a$</td>
<td>2.01 ± 0.44</td>
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<tr>
<td>ECM-F</td>
<td>3.43 ± 0.51$^a$</td>
<td>2.04 ± 0.49</td>
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Data represent means ± S.D. (n = 3).

$^a$ Significant difference compared with ECM scaffold, $P < 0.05$. 

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![Fig. 6](image-url)  
ECM scaffolds derived from mouse fibroblasts. (a) Gross appearance of a mesh-like ECM-mF. Scale bar, 2 mm (b) SEM image of the ECM-mF. Scale bar, 500 µm (c) HE staining revealed that ECM-mF was free of cellular components. Scale bar, 100 µm (d) ATR-FTIR spectra showed the PLGA template was removed from the ECM-mF. (e) DNA contents in the mouse cell-ECM-PLGA constructs and ECM-mF. Data represent means ± SD. ***, significant difference, $P < 0.001$. 

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Therefore, the composition of the ECM scaffolds depends on the cell type and cell phenotype used to prepare the scaffolds. Quantitative analysis of sulfated glycosaminoglycans (sGAG) showed that sGAG remained in the ECM scaffolds (Table 2). However, the sGAG contents decreased after decellularization and template removal. The amount of soluble collagen also decreased after decellularization and PLGA removal (Table 3). These results indicate that the decellularization and PLGA removal processes partially removed some of the extracellular matrices.

The combination of in vitro three-dimensional cell culture in a temporary skeleton and selective removal of the template provides a useful method for preparing ECM scaffolds. The ECM scaffolds showed a subtle porous structure constructed by various ECM biomolecules. Cellular components and PLGA template were removed by decellularization and Na3PO4 treatments. The compositions depended on the cell sources used to fabricate ECM scaffolds. Other than MSCs, chondrocytes and fibroblasts, a large variety of cell types, are thought to be available for preparing their respective ECM scaffolds by the present method. A combination of different cells might offer a better recapitulation of the tissue (organ)-specific ECM [37] and satisfy the complexity requirements of tissue engineering and regenerative medicine [38].

Fig. 7. HE staining of the implanted materials and their surrounding tissues. (b) is the high magnification of (a). aECM, autologous ECM scaffold; Allo, allogeneic ECM scaffold; BCS, bovine collagen sponge; Mu, panniculus carnosus muscle; arrows and areas within the two dashed lines indicate implanted materials. Scale bar = 200 μm in (a) and 100 μm in (b).
3.2. Host tissue responses to aECM scaffold

Mouse autologous ECM (aECM-mF) scaffolds were prepared using autologous mouse fibroblasts, which were isolated from biopsies of each mouse. The ECM-mF scaffolds showed mesh-like three-dimensional structures (Fig. 6a and b). The removal of the cellular components and PLGA templates was confirmed (Fig. 6c and d). The amount of DNA in the ECM-mF was 0.42% of the value before decellularization (Fig. 6e). They primarily consisted of type I collagen, type III collagen, fibronectin, vitronectin, laminin, decorin, and biglycan (Supplementary Fig. 4).

The aECM-mF scaffolds were implanted into a subcutaneous pocket of the respective ICR mouse where the fibroblasts were isolated. The host tissue responses to the aECM scaffolds were compared with those to allogeneic ECM scaffolds (ECM scaffolds prepared from allogeneic mouse cells), xenogeneic bovine collagen sponge (BCS), and PLGA mesh. After implantation for one week, the materials were harvested with the surrounding tissues (Supplementary Fig. 1) for analysis of inflammatory and immune host responses. From the hematoxylin and eosin (HE) staining (Fig. 7a and b), it was found that the host cells had penetrated into the aECM-mF and allogeneic ECM-mF scaffolds. No dense fibrous layers surrounding the implanted materials were observed. However, the BCS and PLGA were “wrapped” by dense fibrous layers without being remodeled [39].

Immunocytochemical staining of the 1-week implants was performed to investigate cellular responses in the host tissue reactions. The macrophages in the implants were stained and counted (Fig. 8a). The sham operation group had very few macrophages. Among the four implanted materials, the lowest number of macrophages was in the aECM-mF scaffolds. The percentages of macrophages/total cells in different groups were compared (Fig. 8b). The lowest proportion of macrophages was found in the aECM group. The macrophage percentage of the allogeneic group was significantly higher than that of the aECM group.

Fig. 8. Micrographs of immunocytochemically stained macrophages (a) and macrophage/total cell percentages in each group (b). aECM, autologous ECM scaffold; Allo, allogeneic ECM scaffold; BCS, bovine collagen sponge; Mu, panniculus carnosus muscle; arrows and areas within the two dashed lines indicate implanted materials. Scale bar = 100 μm (b). Data represent mean ± SD (n = 3). *** significant difference, P < 0.001.
of the aECM scaffolds ($P < 0.001$). The macrophage percentages elicited by the BCS and PLGA scaffolds were significantly higher than were those of the ECM scaffolds ($P < 0.001$). Some neutrophils were observed only in the PLGA, BCS, and allogeneic groups (Fig. 9a).

When biomaterials are implanted, neutrophils and macrophages have been reported to be involved in the inflammatory responses at the implantation site. Neutrophils serve to remove foreign materials and trigger other host responses by secreting factors that summon other immunocytes [23]. And macrophages become activated and act as the main mediators of the host tissue responses. The excretion of soluble mediators by macrophages can influence the behavior of other leukocytes [22,40]. The absence of neutrophils and lowest macrophage percentage indicate aECM-mF induced the lowest inflammatory responses. A few MHC class II antigen-presenting cells were observed in the allogeneic ECM-mF and BCS implantation groups (Fig. 9b). These immunogenic antigen-presenting cells are supposed to exacerbate inflammation and immunological reactions. No T cells were detected in any of the groups after 1-week implantation.

We further investigated the cytokine profiles, which are the key mediators in the host tissue response [22]. The expressions of genes encoding interleukin-10 (IL10), interleukin-2 (IL2), interleukin-4

![Fig. 9. Neutrophils (a) and MHC II-presenting cells (b) in the implanted materials and surrounding tissues revealed by immunocytochemical staining. aECM, autologous ECM scaffold; Allo, allogeneic ECM scaffold; BCS, bovine collagen sponge; Mu, panniculus carnosus muscle; arrows and areas within the two dashed lines indicate implanted materials; circles indicate positive cells. Scale bar = 100 μm.](image-url)
Among the samples, there was no significant difference in the transcription level of (Il10), and tumor necrosis factor-α (Tnf) in the connective tissues under the 1-week implanted materials were analyzed by real-time RT-PCR. The expression of Il10 was lower in the aECM and control groups than it was in the allogeneic ECM, BSC, and PLGA groups (Fig. 10a). Among the samples, there was no significant difference in the transcription level of Tnf although it was lower in the aECM group (Fig. 10b). No Il2 and Il4 gene expressions were detected. These results indicate aECM-mF induced minimal cytokine mediation to modulate the host responses.

All of the results from the host tissue response analyses indicated excellent biocompatibility of the aECM scaffolds, which is essential for ideal tissue engineering scaffolds [17]. Furthermore, the use of autologous serum [41] and serum-free culture [42] is technically possible to reduce the use of animal serum and to minimize the potential side effects induced by exogenous materials [43].

4. Conclusions

A method was developed to prepare aECM scaffolds by combining culture of autologous cells in a three-dimensional template, decellularization, and template removal. The aECM scaffolds showed excellent biocompatibility when implanted. By using aECM scaffolds for the culture of autologous cells, “full autologous tissue engineering” could be realized to make the tissue engineered construct more biocompatible with the host. Furthermore, ECM scaffolds may constitute a potent tool for other biomedical researches such as artifical heart. The full color images can be found in the online version, at doi:10.1016/j.biomaterials.2010.12.016.

Acknowledgement

This work was supported by World Premier International Research Center Initiative on Materials Nanoarchitectonics from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.biomaterials.2010.12.016.

References


