Commercially available materials as scaffold candidates for adipose-derived stromal/progenitor cell tissue engineering

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Received 18 February 2013; received in revised form 14 March 2013; accepted 27 May 2013
Available online 5 February 2014

KEYWORDS
adipose-derived stromal/progenitor cells; scaffold; stem cell

Summary Background: Adipose-derived stromal/progenitor cells (ADSCs) are of great interest because of their unique capacity for prolonged or unlimited self-renewal and their ability to differentiate into multiple lineages. Combining ADSCs with biomaterial scaffolds provides a promising strategy for cellular delivery and tissue engineering.

Objectives: A wide range of biomaterials have been developed for different applications. We here evaluate the current commercially available medical materials as scaffolds in combination with rat ADSCs for tissue engineering applications.

Methods: Experiments (anchorage independent growth, gap junctional intercellular communication, multilineage differentiation, cell-surface marker, and reverse transcription polymerase chain reaction) were performed to prove these primary cultured cells had the same characteristics as ADSCs. Twelve kinds of medical materials were then used as scaffolds to see which ADSCs can grow and develop.

Results: The cultured Wistar rat cells had the characteristics of stem cells, including a high frequency of anchorage-independent growth in soft agar and a lack of gap junctional intercellular communication in cell type with serpiginous morphology. These cells can be differentiated into adipocytes and osteocytes after induction. The differentiated phenotypes were verified by morphology, special stains, as well as detection of tissue-specific mRNA. In addition, these ADSCs could grow into the Kaltostat, CollaWound, Tissucol Duo Quick (Baxter), and Spongostan Dental after 144 hours incubation.

Conflicts of interest: The authors have no conflicts of interest relevant to this article.

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http://dx.doi.org/10.1016/j.fjs.2013.06.006
1. Introduction

Adipose-derived stromal/progenitor cells (ADSCs) were first described by Zuk et al. and have the capacity for prolonged or unlimited self-renewal and ability to differentiate into multiple lineages. It can achieve lower donor site morbidity. Compared with other sources of mescenchymal stem cells, we believe that ADSCs are the best choice for clinical use because they are easier to harvest from adipose tissue than bone marrow and there are plenty of fat sources in the human body. Moreover, there are fewer ethical issues in using cells from fat tissue than those harvested from human embryos. Therefore we studied the ADSCs as the material instead of hematopoietic stem cell transplantation.

Development of scaffolds has demonstrated promise for tissue engineering applications and can be used as replacements for lost or damaged tissues. Such biomaterial scaffolds provide a three-dimensional structure for stem cells to be implanted to develop tissue. A wide variety of natural and synthetic materials and composite mixtures of materials have been investigated as scaffolds for tissue regeneration. As plastic surgeons do not have access to chemical techniques to invent new material or the ability to complete the complicated Phase I/II/III clinical trials for new human use material, we used medical materials that have already been approved for human use. We evaluated the current commercially available medical materials as scaffolds in combination with rat ADSCs to verify their biocompatibility.

2. Materials and methods

2.1. Harvesting of fat tissue from rats

Male Wistar rats (n = 6) weighing 300–400 g were used in this study. The animal use protocol listed below has been received and approved by the Institutional Animal Care and Use Committee (IACUC). After rats were anesthetized by intraperitoneal injection of Zoletil 50 (Virbac Taiwan Co, Ltd, Taipei, Taiwan), inguinal skin was prepared and subcutaneous fatty tissue around 10 g was resected from the right or left inguinal area. These adipose tissues were rinsed with D-phosphate-buffered saline (D-PBS) to remove the majority of erythrocytes. Tissues were scraped into a tube, washed in D-PBS and centrifuged at 750 g for 5 minutes. The preparation procedures were as described by Lin et al. The upper fraction was transferred to another tube using a sterile pipette and washed. After centrifugation, the tissue was distributed into tubes containing Dulbecco’s modified Eagle medium (DMEM) with 1 mg/mL collagenase, 2 mM n-acetylcysteine, and 0.2 mM ascorbic acid 2-phosphate. After incubation at 37°C for 3 hours, tubes were centrifuged at 750g for 5 minutes to remove the collagenase solution and the pellet was washed and then incubated in DMEM with 10% fetal bovine serum (FBS), 2 mM NAC, and 0.2 mM ascorbic 2-phosphate in a 5% CO2 incubator. After 24 hours, the unattached cells were removed by washing with PBS. Then, 5 mL of K-NAC medium with 5% FBS were added to each 25-cm² flask. The medium was changed every other day until confluence. These cells were generated for trypsinization and stored in liquid nitrogen or kept for subculturing.

2.2. Anchorage independent growth

A total of 50,000 cells in 3 mL of 0.33% agarose medium were plated on top of 3-mL of prehardened 0.5% agarose medium in each of triplicate dishes (6 cm). Then, 2.5 mL of liquid medium (K-NAC with 5% FBS) were added and renewed once every 3 days. The number of colonies developed were scored under a microscope with the dish containing anchorage independent growth colonies on the top of a dish with grids. The preparation procedures were as described by Lin et al.

2.3. Gap junctional intercellular communication

The gap junctional intercellular communication (GJIC) was studied by scrape loading/dye transfer technique. The dye transfer between cells was observed using a Nikon Eclipse TE300 UV fluorescence microscope; Tokyo, Japan and recorded by a digital camera connected to a computer. The preparation procedures were as described by Lin et al.

2.4. Multilineage differentiation: adipogenesis and osteogenesis

For differentiation induction of putative ADSCs into adipocytes and osteoblasts, the cells were initially developed and propagated by different supplementations in DMEM, following the induction procedure reported by Lin et al.

2.5. Reverse transcriptase-polymerase chain reaction

Reverse transcription (RT) of the RNA was performed in a 20-µL reaction mixture using a poly-T primer with 20 U SuperScript, 10 mM dNTP mix, 0.5 mM bovine serum albumin, 1 M dithiothreitol, and supplied buffer. The mixture was incubated at 42°C for 1 hour and at 94°C for 10 minutes to inactivate the RT. One-tenth of this reaction was added to a standard polymerase chain reaction (PCR) mixture with tissue-specific primers, dNTP mix, bovine serum albumin, Taq buffer, and Taq polymerase. PCR products were

Conclusion: Kaltostat, CollaWound, Tissucol Duo Quick (Baxter), and Spongostan Dental have better biocompatibility and can be considered as scaffold materials for future clinical research.

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resolved on a 2% agarose gel stained with ethidium bromide. The preparation procedures were as described by Tholpady et al.16

2.6. Fluorescence-activated cell sorting analysis of ADSCs

In order to determine the characteristics of these primary cultured cells, the ADSCs within three passages were harvested by trypsinization, and then the cells were fixed in neutralized 2% paraformaldehyde solution for 30 minutes. The fixed cells were washed twice with PBS and incubated with antibodies against CD90, CD29, CD34, CD31, and CD45 for 30 minutes. Primary antibodies were directly conjugated with fluorescein isothiocyanate (FITC) and phycoerythrin. For isotype control, nonspecific FITC-conjugated immunoglobulin G was substituted for the primary antibodies.

2.7. Verification of scaffold biocompatibility

Tissue engineering holds promise in medicine by combining scaffolds, cells, and growth factors. The tissue engineering implant can also be integrated into the existing tissue, resulting in a seamless transition between the two. This advantage can be mostly attributed to the development of biomimetic scaffolds. Using the current available medical materials, we tested whether the ADSCs can grow into the three-dimensional environment that scaffolds provide. We tested 12 different kinds of scaffold including Seprafilm (Genzyme Corporation, Cambridge, MA, USA), Inion (AESCUPL Implant System Inc., 3773 Corporate Parkway Center Valley, PA, USA), CollaWound (2nd Floor, No. 360, Ruiguang Road, Neihu, Taipei, TW), Intrasite Gel (Smith and Nephew Medical LTD., 101 Hessle Rd., Hull, UK), DuoDERM (Conva Tec, Division of E. R. Squibb & Sons, L.L.C., Princeton, NJ, USA), Surgicel (Johnson & Johnson Medical Ltd., UK), Kaltostat (Britcair, Aldershot, Hants, UK), Medpor (Pores Surgical Inc., College Park, GA, USA), and Tissucol Duo Quick (Baxter AG, Vienna, Austria) (Table 1). Bolus injection of cell suspension (5 $\times$ 10^6 cells/mL) into these scaffolds were performed and cultured in a 5% CO2 incubation for 24 hours. The preliminary data showed that ADSCs could grow into Kaltostat, Tissucol Duo Quick, CollaWound, and Spongostan Dental. Therefore, a second experiment was performed. We used commercially available Qtracker (Invitrogen, Inc., Hayward, CA, USA) to label the ADSCs. Then, 5 $\times$ 10^6 cells/mL ADSCs were bolus injected into these four scaffolds. On Day 3 and Day 6, fluorescence microscopy was used to observe these cells. Live cells labeled with bioconjugated quantum dots (QDs) can be readily imaged by fluorescent microscopy.17

3. Results

3.1. Development of cell culture from rat adipose tissue

After 3 hours, the culture of minced adipose tissue in the DMEM with 10% FBS showed most of the cells remaining in

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**Table 1** Twelve commercially available medical materials.

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Bioabsorbable</th>
<th>ADSCs growth</th>
<th>Clinical use</th>
<th>Implant or screw reduction of post-surgical adhesions</th>
<th>Wound care product to promote wound healing and new tissue development</th>
<th>Surgical Fibrillar?</th>
<th>Surgicel?</th>
<th>DuoDERM®</th>
<th>Spongostan Dental?</th>
<th>Kaltostat®</th>
<th>Medpor®</th>
<th>Tissucol Duo Quick®</th>
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<td>Seprafilm®</td>
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12 types of commercially available medical materials were tested whether the ADSCs could grow into the scaffolds provided. The preliminary data showed that ADSCs could grow into 7 out of 12 scaffolds. Therefore, a second experiment was performed. We used commercially available Qtracker (Invitrogen, Inc., Hayward, CA, USA) to label the ADSCs. Then, 5 $\times$ 10^6 cells/mL ADSCs were bolus injected into these four scaffolds. On Day 3 and Day 6, fluorescence microscopy was used to observe these cells. Live cells labeled with bioconjugated quantum dots (QDs) can be readily imaged by fluorescent microscopy.
suspension, including many erythrocytes. After washing procedures, few attached cells proliferated actively and became confluent after 1 week in 25-cm² flasks. These putative ADSCs appeared serpiginous or fibroblast-like appearance microscopically11 (Fig. 1A and B).

3.2. Verification of rat ADSCs

3.2.1. Anchorage independent growth

Stem cells and cancer cells are capable of growing in soft agar; by contrast, ordinary cells are not able to grow on soft agar.10,18 The result showed these rat adipose-derived cells can undergo anchorage-independent division to form a colony on soft agar (Fig. 1C), whereby we can differentiate stem cells from fibroblasts.11

3.3. Induction of multilineage differentiation

3.3.1. Adipogenesis

After treatment with 3-isobutyl-1-methylxanthine, dexamethasone, indomethacin, and insulin for 3 days and insulin for 1 day, lipid-producing adipocytes appeared after one cycle of treatment.10,15 The lipid vacuoles became larger after two or three cycles of treatment and were clearly visible without staining under a microscope (Fig. 1D). The ability of these cells to differentiate into adipocytes is similar to ADSCs.11

3.3.2. Osteogenesis

After treatment with dexamethasone, Asc-2P, and β-glycerophosphate in D-medium for 2–4 weeks, the cell monolayer was covered with a layer of visible deposits. Through

Figure 1  Under (A) 40× and (B) 400× magnification, the morphology is serpiginous or has a fibroblast-like appearance, which is similar to human adipose-derived stromal/progenitor cells. (C) Under 200× magnification, one can see cells undergo anchorage-independent division to form a colony on soft agar. (D) With adipogenic differentiation, the bright lipid vacuoles are clearly visible without staining under 100× magnification. (E) With osteogenic differentiation, deposits of calcified extracellular matrix are clearly seen with Alizarin Red staining under 100× magnification.

Figure 2  After 3 hours, strong Lucifer Yellow dye is still retained in the gap junction intercellular communication-deficient serpiginous cells with fluorescent microscopy.
Alizarin Red staining, it has shown that these deposits are calcified extracellular matrix (Fig. 1E), which indicates that these cells retain the ability of osteogenic differentiation.

3.3.3. GJIC
Cancer cells and many adult human stem cells have been shown to have GJIC. The ADSCs were studied by Lucifer Yellow scrape loading/dye transfer technique. The result shows these serpiginous cells were deficient in dye transfer (Fig. 2A and B). This characteristic shows that these cells are similar to ADSCs.

3.3.4. RT-PCR
Undifferentiated ADSCs were induced to differentiate through the adipogenic or osteoblastic pathways, and were then analyzed for the presence of tissue-specific mRNA by RT-PCR. Both cell types were assayed after 4 weeks. Specific markers for these cell types were peroxisome proliferator activated receptor γ (adipocytes) and osteonectin (osteoblasts).

In the RT-PCR analysis, ADSCs grown in adipogenic media were positive only for peroxisome proliferator activated receptor γ (Fig. 3A), but not for other markers. When treated with osteogenic media, ADSCs were positive for osteonectin (Fig. 3B). The PCR results were consistent with the morphology and cell-type-specific staining in determining the differentiated cell type.

3.4. Fluorescence-activated cell sorting
Fluorescence-activated cell sorting analysis of rat ADSCs (3 passages) using mouse monoclonal antibodies: specific FITC and phycoerythrin conjugated antibodies against surface markers. An isotype control was included in each test (gray lines). The result showed that they expressed CD90, CD34, and CD29 markers but did not express CD31 and CD45 markers (Fig. 4). Fluorescence-activated cell sorting analysis of surface markers was consistent for mesenchymal stem cells. This result was compatible with other reports.

3.5. Verification of scaffold biocompatibility
Bolus injection of the suspension of ADSCs (5 x 10^6 cell/mL) into the scaffold was performed. The scaffolds were immersed with culture medium and put in a 5% CO2 incubator for 24 hours. Cells were able to attach to and form a three-dimensional structure in Kaltostat, Tissucol Duo Quick, CollaWound, and Spongostan Dental (Fig. 5). However, ADSCs were unable to attach to the other materials and cell death was observed (Fig. 6). QD signals were detected in Kaltostat (Fig. 7), Tissucol Duo Quick (Fig. 8), CollaWound (Fig. 9), and Spongostan Dental (Fig. 10) at 72 hours and 144 hours.

4. Discussion
Ideal repair after soft tissue injury is a challenge in plastic reconstruction surgery. Using a patient’s own mesenchymal stem cells to regenerate the tissue offers a new solution to restore soft tissue defect with less donor site morbidity. Concerning the cell source, fibroblasts are important in collagen synthesis and remodeling, but the main disadvantages are their limited lifespan, capacity for proliferation, and narrow usage due to their differentiated state. Adipose tissue, however, is an abundant and accessible source of adult stem cells that can be collected under local anesthesia with less donor site morbidity. Immunomodulatory capacity and less immune reaction make ADSCs a greater potential for transplantation. Collectively, these beneficial features together with a lack of ethical concerns have promoted ADSCs to the forefront of tissue engineering.

We successfully isolated a group of Wistar rat cells from the subcutaneous fatty tissue and proved that these cells have the characteristics of ADSCs. Creating and maintaining the shape and dimensions of the engineered soft tissue are perhaps best accomplished by scaffold biomaterials. Most scaffolds provide a three-
dimensional environment in which tissue can grow and develop. ADSCs can be expanded in culture and introduced into scaffolds to form tissues prior to or after differentiation. The engineered tissue structure can then be inserted into the body to repair the soft tissue defect. For example, calcium phosphate has been used for bone tissue engineering because of its pore network and ability to bond with bone. Hydrogel is regarded as an injectable scaffold to introduce cells into the solution prior to gelation.

Three-dimensional scaffold has been considered as a key factor to obtain high differentiation, proliferation rate, and growth stability. However, it is a challenge to find an appropriate scaffold that should promote cell adhesion, proliferation, and differentiation and at the same time not develop an immune reaction. A wide range of biomaterials has been developed for different applications. However, the Phase I/II/III clinical trials for developing human-use materials are very complicated and may need more than 10 years to complete. To avoid expensive and time-consuming clinical trials in humans for testing the biocompatible scaffolds, we choose 12 kinds of current commercially available medical materials to verify their biocompatibility. We found that ADSCs could grow into Kaltostat, CollaWound, Tissucol Duo Quick, and Spongostan.

Figure 5  Under 200× magnification, cells can be seen to be attached to the following materials and forming three-dimensional structures: (A) Kaltostat; (B) Tissucol Duo Quick; (C) CollaWound; (D) Spongostan Dental at 24 hours.

Figure 6  Under 100× magnification, no cells attached to or formed three-dimensional structures and cell death is observed in the following materials: (A) Seprafilm; (B) Medpor; (C) DuoDERM; (D) Surgicel Fibriller; (E) Intrasite Gel; (F) Surgicel Nu-Knit; (G) Inion plate; (H) Surgicel.
**Figure 7** Kaltostat. (A) Bright-field and (B) fluorescent images of the same view show adipose-derived stromal/progenitor cells labeled with quantum dots (red spots) at 72 hours. (C) Bright-field and (D) fluorescent images of the same view show adipose-derived stromal/progenitor cells labeled with quantum dots (red spots) at 144 hours. All magnifications: 40×.

**Figure 8** Tissucol Duo Quick. (A) Bright-field and (B) fluorescent images of the same view show adipose-derived stromal/progenitor cells labeled with quantum dots (red spots) at 72 hours. (C) Bright-field and (D) fluorescent images of the same view show adipose-derived stromal/progenitor cells labeled with quantum dots (red spots) at 144 hours. All magnifications: 40×.
Figure 9  CollaWound. (A) Bright-field and (B) fluorescent images of the same view show adipose-derived stromal/progenitor cells labeled with quantum dots (red spots) at 72 hours. (C) Bright-field and (D) fluorescent images of the same view show adipose-derived stromal/progenitor cells labeled with quantum dots (red spots) at 144 hours. All magnifications: 40×.

Figure 10  Spongostan (A) Bright-field and (B) fluorescent images of the same view show adipose-derived stromal/progenitor cells labeled with quantum dots (red spots) at 72 hours. (C) Bright-field and (D) fluorescent images of the same view show adipose-derived stromal/progenitor cells labeled with quantum dots (red spots) at 144 hours. All magnifications: 40×.
Dental after 144 hours’ incubation. Kaltostat is a calcium sodium alginate made from brown seaweed and provides a moist healing environment and hemostasis. CollaWound is composed of pure collagen and provides a provisional matrix in the wound cavity to act as a scaffold for the ingrowth of cells involved in normal healing process. Tissucol Duo Quick is based on the final stage of coagulation, specifically the conversion by thrombin, factor XIII, and calcium of a fibrinogen monomer into a fibrin polymer, to create a hemostatic adhesive clot. Spongostan Dental is an absorbable gelatin sponge with a hemostatic effect for surgical and dental purposes. These four biomaterials are commercially available, nonallergic to patients, biocompatible, and easily packed into the defect site; all can be regarded as good material candidates for future experiments.

4.1. Study limitations

This is a preliminary report for testing the potential scaffolds that can be used in humans. However, several limitations in the design of this study can be identified. First, our results only showed the early cell-scaffold response rather than any long-term tissue changes and formation. The growth rate and proliferation potential of ADSCs in these scaffolds were not specifically studied. Second, because the total amount of Qtracker is a constant number, further proliferation of ADSCs will dilute the density of Qtracker. Thus, this study design is not able to show the quantity change of ADSCs in these scaffolds. Third, the Qtracker can only be detected within 2 weeks; we need a different ADSC tracking technique to observe the position and quantity of ADSCs for more than 2 weeks. Furthermore, cell viability and differentiation in these scaffolds will need further investigation by immune staining techniques in future studies. The conclusions should be interpreted with these limitations in mind.

5. Conclusion

We successfully isolated rat ADSCs and then verified the biocompatibility potential of available scaffold’s. Kaltostat, CollaWound, Tissucol Duo Quick (Baxter), and Spongostan Dental have good biocompatibility and can be considered as scaffold materials for future clinical research.

Acknowledgments

This project was supported by the KMUH 98-8R22 grant.

References


