

Simple and Longstanding Adipose Tissue Engineering in Rabbits

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Abstract

Adipose tissue engineering for breast reconstruction can be performed for patients who have undergone breast surgery. We have previously confirmed adipogenesis in mice implanted with type-I collagen sponge with controlled- release of fibroblast growth factor 2 (FGF2) and human adipose tissue-derived stem cells. However, in order to use this approach for treating breast cancer patients, FGF2 is not readily available and a large size of adipose tissue is needed. Herein, we aimed to regenerate large amounts of adipose tissue without FGF2 for a long period. Under general anesthesia, cages made of polypropylene mesh were implanted into the rabbits' bilateral fat pad. The size of cage was 10 mm in radius and 10 mm in height. Minced type-I collagen sponge was injected as a scaffold into the cage. Regenerated tissue in the cage was examined with ultrasonography, and the cages were harvested 3, 6, and 12 months after the implantation. Ultrasonography revealed a gradually increasing homogenous high echo area in the cage. Histology of the specimen was assessed with hematoxylin-eosin staining. Percentages of regenerated adipose tissue area were $76.2 \pm 13.0\%$ and $92.8 \pm 6.6\%$ at 6 and 12 months after the implantation, respectively. Our results showed *de novo* adipogenesis 12 months after the implantation of only type-I collagen sponge inside the space. Ultrasonography is a non-invasive and useful method

to assess the growth of the tissue inside the cage. This simple method could be a promising clinical modality in breast reconstruction.

Introduction

Breast cancer is common disease among women worldwide. Breast surgery is not the best, but one of the local treatments of breast cancer since any defect of breast is distressing for patients. There are many ways for breast reconstruction, but each technique has its drawbacks. For example, silicone implants can induce foreign body response and be an obstacle during breast cancer screening. Autologous tissue graft implantation is a long surgical procedure, and results in operation scars at many sites. Autologous fat transfer has the drawbacks of shrinkage, fat necrosis or oil cyst formation [1]. Hence, adipose tissue engineering for breast reconstruction is a fourth alternative for patients who have undergone breast surgery.

Previous study has confirmed adipogenesis in mice implanted with a type-I collagen sponge with controlled- release of FGF2 and human adipose tissue-derived stem cells (ASCs) [2]. We showed that human ASCs not only function as progenitor cells for *in vivo* adipogenesis but also induce *de novo* adipogenesis in type I collagen scaffold. ASCs are adipose tissue-specific progenitor cells and differentiate into adipocytes or vascular cells. Type I collagen exists widely in the animal body and exhibits good biocompatibility, gelatification, and structural stability. Hence, type I collagen is a superior material for use in regenerative medicine [3, 4] [5]. FGF2 enhances

adipogenesis [6, 7]. However, FGF2 is not readily available for breast cancer patients; furthermore, a large size of adipose tissue is needed for a long period. In addition, some diagnostic modalities are needed during follow up of the implant. The aim of this study is to regenerate large amounts of adipose tissue without FGF2 and to maintain for a long period. Another aim of this study is to evaluate the usefulness of USG as a diagnostic modality.

Materials and methods

Materials

A polypropylene (PP) mesh was purchased from FLON Industry Inc. (Tokyo, Japan). We prepared a column cage made of PP mesh with a pore size of 200 μm and 5-0 prolene strings (Ethicon Inc, New Brunswick, NJ, USA). The radius of the cage was 10 mm, and the height was 10 mm. PELNAC, which is approved for artificial dermis, consisting of an atelocollagen sponge layer and a silicone layer was kindly supplied by Gunze Co Ltd (Kyoto, Japan). The sheet was 82 x 60 mm in size. First, the silicone layer of PELNAC was removed. Then, the type I collagen layer was minced, and suspended with 8 mL of natural saline (Otsuka pharmaceuticals Co Ltd, Tokushima, Japan) in a 10-mL syringe.

Animal experiments

All the animal experiments were reviewed by the Committee on the Ethics of Animal Experiments (Faculty of Medicine, Kyoto University, Kyoto, Japan), and were performed in accordance with the Guidelines for Animal Experiments of the Faculty of Medicine, Kyoto University.

Female New Zealand white rabbits (2.5-3.0 kg; Shimizu Laboratory Supply, Kyoto, Japan) were used. General anesthesia was applied with an intramuscular injection of midazolam (4 mg/kg), medetomidine hydrochloride (0.16 mg/kg), and butorphanol tartrate (0.4 mg/kg). A PP cage was implanted into New Zealand white rabbit's bilateral fat pad, and the suspended type I collagen sponge was injected into the cage with 16G intravenous cannula. Finally, the skin was closed with a buried suture. The implant was harvested 3, 6, and 12 months after implantation, and every specimen was assessed with hematoxylin-eosin (H-E) stain. Every experimental group consisted of 4 specimens. Percentage of regenerated adipose tissue area within a cage was calculated with NIH Image J software.

Ultrasonographic observation

HITACHI MyLab25 (Hitachi medical corporation, Tokyo, Japan) was used, and ultrasonography (USG) was performed under general anesthesia at 1 week, 1 month, 3 months, 6 months, and 12 months after implantation.

Statistical analysis

Statistical differences were determined with a Wilcoxon test. A statistical significance was assigned as $*p<0.05$. JMP version 9 software (SAS Institute Inc., Cary, NC, USA) was used for statistical analysis.

Results

Ultrasonographic findings

The ultrasonographic findings are shown in Figure 2. An anechoic area and internal hyperechoic mass were noted inside the cage at 1 week (Fig 1A) and 1 month after implantation (Fig 1B). A homogenous high echo area was detected inside the cage after 3 months until 12 months (Fig 1 C, D, and E).

Cross section observation

Figure 1F and 1G show the gross sections at 3 and 6 months after implantation. At 3

months after implantation, the appearance of the inner side the cage showed high water content (Fig 1F). At 6 months after the implantation, the appearance of the inner side of the cage became whitish compared to that of the specimen at 3 months (Fig 1G).

H-E staining

Figure 1 shows H-E staining of the specimen. At 3 months after implantation, type I collagen scaffold was observed mainly inside the cage under low magnification (Fig 1I). Under high magnification, several vacuoles were found mainly at the margins of the cage (Fig 1L). These vacuoles were morphologically adipocytes. Further, many nuclei were seen among the scaffold. At 6 months, adipose tissue occupied a large part of the cross-section surface under low magnification (Fig 1J). Under high magnification, large amount of mature adipocytes were observed and angiogenesis was noted (Fig 1M). At 12 months, mature adipose tissue was observed mostly inside the cage (Fig.1K). Complete regeneration of the adipose tissue was achieved inside the cage (Fig 1N).

Percentage of regenerated adipose tissue area

Average percentages of regenerated adipose tissue area within a cage were $31.7 \pm$

10.3, 76.2 ± 13.0 , and $92.8 \pm 6.6\%$ at 3, 6, and 12 months after implantation, respectively (Figure 2). Regenerated adipose tissue area at 6 and 12 months were significantly increased compared to at 3month after implantation ($p=0.03$).

Discussion

Tissue engineering requires a cell source, a scaffold, and growth factors [8]. In line with these requirements, we previously confirmed adipogenesis in mice by implanting ASCs with type I collagen scaffold with a controlled- release of FGF2 [2]. Further, we confirmed *in situ* adipogenesis in rabbits by implanting type I collagen with the controlled- release of FGF2. Repeated administration of the mixture of collagen scaffold and gelatin microspheres containing FGF2 enabled significantly higher area at 3months after implantation than that of the single administration of each component [9]. In this study, implanting type I collagen inside the cage without ASCs and controlled- release of FGF2 resulted in adipogenesis. And an average percentage of regenerated adipose tissue within the cage was $92.8 \pm 6.6\%$ at 12 months after implantation. Type I collagen sponge implanted into the PP cage recruited adequate cell source and necessary growth factors. Fibroblast growth factor 2 is a potent angiogenic factor, known to be effective for adipogenesis [6, 7]. However, FGF2 cannot

be clinically used for treating malignant diseases, because FGF2 has a potency of breast carcinogenesis [10]. Our method of adipose tissue engineering without FGF2 is a promising way to use FGF2 for clinical purposes.

Some mechanisms of adipogenesis have been reported. First, type I collagen sponge is known to be superior scaffold for ASC adhesion because of its porous structure [5] [7]. Generally, cells cannot survive in the absence of adhesive materials. So, the observation in this study that lots of nuclei with recruited cells were observed in the scaffold 3 months after implantation suggested that these cells were precursors of mature adipocytes and adhered to type I collagen sponge. Collagen degradation is essential for cell migration, proliferation, and differentiation. Type I collagen scaffold have degraded and mature adipose tissue was generated inside the cage 6 months after implantation. Mature adipose tissue still remained 12 months after implantation. O'Connor et al reported a higher rate of adipogenic differentiation *in vitro* with type I collagen than with fibronectin [11]. We have confirmed type I collagen was excellent scaffold for adipogenesis *in vivo*. Secondly, the fat pad was an excellent implantation site for adipogenesis. Because the fat tissue in this study was healthy, optimal conditions for adipogenesis were satisfied. ASCs are present in the adipose tissue and are derived from the surrounding tissue. For example, administration of cell source or

growth factors might be required for efficient adipogenesis when the fat tissue have received radiation therapy. Third, the growth factors required for adipogenesis were produced after implantation. Several growth factors are produced at the operation site after surgery. Soluble FGF2, vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), epidermal growth factor, or insulin-like growth factor-1 (IGF-1) have been detected in the wound drainage fluid [12]. Fibroblast growth factor 2 and PDGF promote *in vitro* ASCs proliferation [13]. Fibroblast growth factor 2 and VEGF are potent angiogenic factors. Angiogenesis enables blood supply and oxygen to the scaffolds. Blood vessel formation was confirmed since 6 months after implantation in this study. Adipose tissue engineering requires *in vivo* vascularization, and *in vivo* vascularization enhanced the formation of engineered adipose tissue. The growth factors emanated after the surgery and contributed to adipogenesis.

The reconstruction of volume-stable adipose tissue for a long period is a great challenge. Although the subcutaneous environment is relatively static, the skin does exert a compressive force on any material placed subcutaneously. It might also be necessary to maintain some extra spaces in order to accommodate the volume-stable adipose tissue [14]. In this study, PP mesh was used to maintain this space and is a landmark of the engineered adipose tissue area. Although it is easy to detect PP mesh

with USG, its material is unabsorbable and hard for breast reconstruction. Further studies will be needed to identify soft and absorbable materials that can be used to maintain extra space in order to apply this technique in clinical situations.

It is important to evaluate the regenerated adipose tissue. We used USG to follow up the implant over time. Regenerated tissue can be distinguished from areas with water content or empty spaces. USG is usually used for breast examination, because it is easy, non-invasive, does not involve radiation-exposure, and is cost-effective as compared to computed tomography, mammography, or magnetic resonance imaging. Herein, USG is a useful modality to assess engineered adipose tissue.

Conclusion

We achieved complete regeneration of adipose tissue inside the cage at 12 months after implantation only by implanting a type-I collagen sponge inside the space. With USG, an anechoic area and internal hyperechoic mass gradually disappeared and were replaced with homogenous high echoic area, reflecting adipogenesis. USG is a non-invasive and useful method to examine the contents inside the cage.

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Figure legends

Fig 1. USG findings 1 week (A), 1 (B), 3 (C), 6 (D), and 12 months (E) after implantation. Gross views of the implant 3 (F), 6 (G), 12 months (H) after implantation. H-E staining (x4) at 3 (I), 6 (J), and 12 months (K) after implantation. H-E staining (x20) at 3 (L), 6 (M), and 12 months (N) after implantation. The scale bars in (L), (M), and (N) indicate 500µm.

Fig 2. Percentages of regenerated adipose tissue area within a cage at 3, 6 and 12

months after implantation. Data are mean $\pm$ standard deviation from n=4, statistical differences (*p < 0.05).

Fig 1

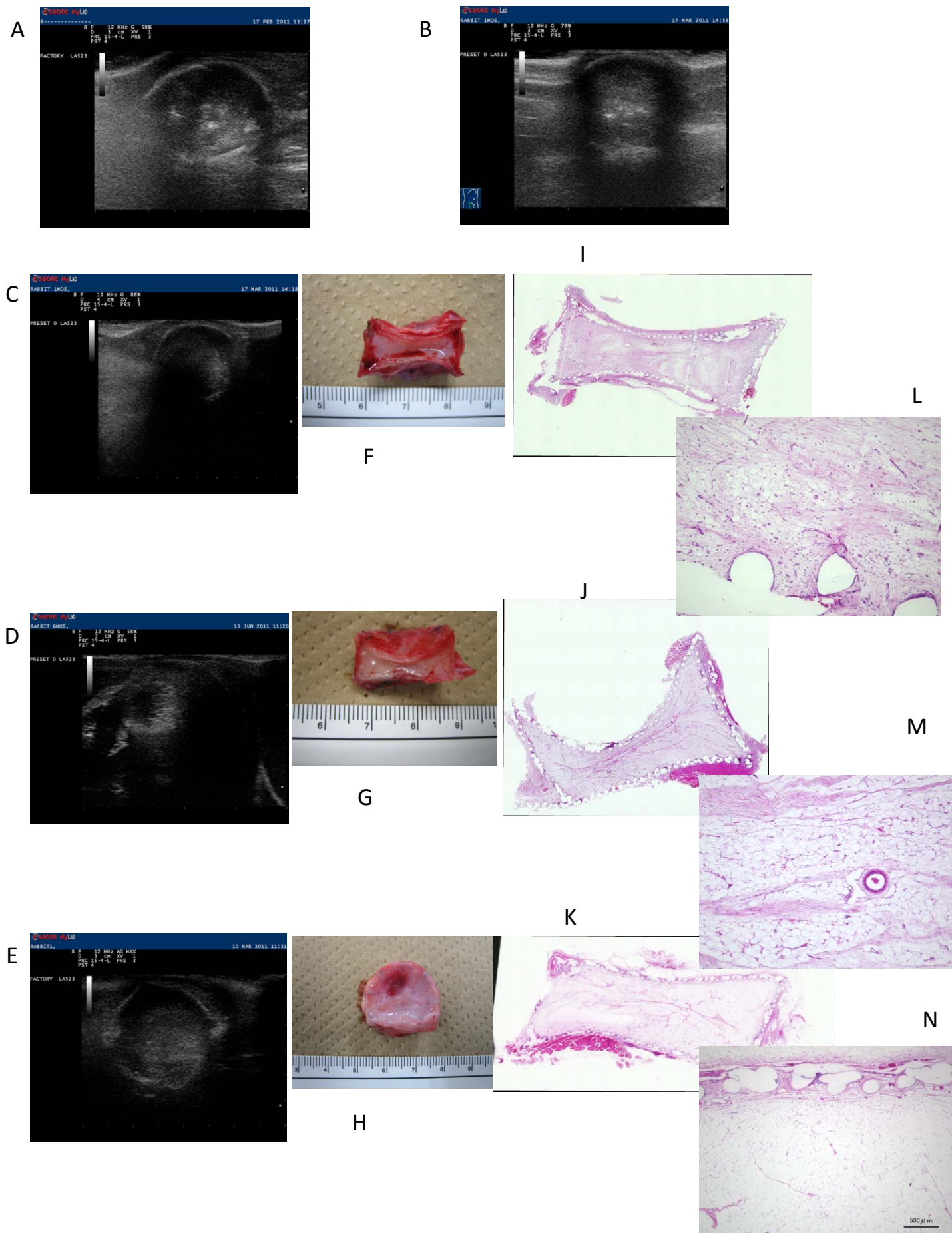


Figure 2

