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A Hydrogel Material for Plastic and Reconstructive Applications Injected into the Subcutaneous Space of a Sheep

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ABSTRACT

Soft tissue reconstruction using tissue-engineered constructs requires the development of materials that are biocompatible and support cell adhesion and growth. The objective of this study was to evaluate the use of macroporous hydrogel fragments that were formed using either unmodified alginate or alginate covalently linked with the fibronectin cell adhesion peptide RGD (alginate-RGD). These materials were injected into the subcutaneous space of adult, domesticated female sheep and harvested for histological comparisons at 1 and 3 months. In addition, the alginate-RGD porous fragments were seeded with autologous sheep preadipocytes isolated from the omentum, and these cell-based constructs were also implanted. The results from this study indicate that both the alginate and alginate-RGD subcutaneous implants supported tissue and vascular ingrowth. Furthermore, at all time points of the experiment, a minimal inflammatory response and capsule formation surrounding the implant were observed. The implanted materials also maintained their sizes over the 3-month study period. In addition, the alginate-RGD fragments supported the adhesion and proliferation of sheep preadipocytes, and adipose tissue was present within the transplant site of these cellular constructs, which was not present within the biomaterial control sites.

INTRODUCTION

MUCH RESEARCH HAS FOCUSED on tissue-engineered constructs for the replacement of skin,[1,2] cartilage,[3–6] bone,[7,8] liver,[9–12] and other tissues. However, little research has involved the development of cell-based biomaterials for reconstruction of soft tissue defects caused by trauma and/or cancer. The goal of using a cell-based biomaterial for reconstructive surgery is to create tissue from transplanted cells that will mimic the shape, structure, and tactile properties of the surrounding native tissue. Cell-based biomaterials

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have the potential to replace the materials currently used such as silicone implants,[13,14] type I collagen for tissue bulking,[15,16] and autologous tissue used for free muscle flaps.[17,18] These approaches have various complications, including (1) the formation of a fibrotic capsule[14] and possible autoimmune complications;[14] (2) the absorption of the type I collagen;[15] and (3) the complex surgical procedure of the muscle flap with possible tissue necrosis.[18]

Successful biomaterial constructs for soft tissue engineering must support cellular adhesion, promote rapid vascular and tissue ingrowth throughout the implant site, and induce a minimal fibrotic capsule and inflammatory response. In addition, it is preferable for the material to be delivered using a minimally invasive procedure. Several approaches have been developed toward creating biomaterials that support cell adhesion.[19–22] However, none of these materials have been used for the delivery of cells for the creation of a soft tissue.

Our group has focused on using a hydrogel material that supports the delivery of cells such as preadipocytes and aids in establishing long-term integration of the cellular implant with the surrounding host tissue. Our previous work described the use of an alginate (a naturally derived hydrogel) material that is covalently modified with a fibronectin cell adhesion peptide, arginine, glycine, and aspartic acid (RGD).[23] This material was manufactured into a macroporous bead that supported cell attachment, adhesion, and proliferation.[24] Small animal studies performed over 6 months demonstrated that the material implanted into a subcutaneous space supported tissue ingrowth with a minimal capsule surrounding the implant, and a minor inflammatory response.[25] In addition, the material retained its shape over the time of the experiment.

To determine whether this material was useful for clinical applications, we conducted a large animal study using a sheep model. Our goal was to determine whether injectable porous hydrogel fragments integrated with the surrounding subcutaneous tissue and whether the tissue formed within the materials was dependent on the presence of the RGD peptide. An additional objective was to demonstrate whether the alginate-RGD porous fragments supported sheep preadipocyte cellular adhesion and growth and whether these cell-based fragments supported the development of new adipose tissue within the transplanted site. It is our goal to use data generated from these and our previous experiments to develop cell-based biomaterials that can be used for the reconstruction of soft tissue defects.

MATERIALS AND METHODS

Biomaterial processing

Ultrapure alginate (medium viscosity [MV]; Pronova, lot no. 411-256-05, Portsmouth, NH) was either covalently modified with the GGGGRGDY peptide sequence[23] or used in an unmodified form. The alginate material was then processed into porous beads as previously described.[24,26] Fragments were made by placing 50-mL porous beads/250 mL of deionized water mixture into a blender (Osterizer Galaxie, Oster, Inc.) and processed using the chop speed for approximately 30 sec. The fragments were sieved using a 425-μm pore filter (no. 40, 35-mesh filter; Fisher Scientific, Pittsburgh, PA), and then removed from the filter (consisting of approximately 500 μm in dimension), and placed into 50-mL conical tubes, each containing 10 mL of fragments in 30 mL of 0.05 M calcium chloride. The tubes containing the fragments were sterilized using a 1.5-Mrad dose (University of Michigan, Gamma Irradiation Facility). Figure 1a is a confocal microscope image of a fragment that has been stained with eosin (Leica SPF Confocal Laser Scanning Microscope, 5× magnification, Leica Microsystems, Inc., Exton, PA).

Animal surgery

Adult domesticated female sheep were used for this study. All experimental procedures were performed in an AAALAC-approved vivarium following Institutional Animal Care and Use Committee guidelines. Each sheep had two surgical procedures. The first procedure was a mini-laparotomy to harvest adipose tissue from the omentum. Isoflurane was used as a general anesthetic.

The second surgical procedure, which took place at least 6 weeks after the initial tissue harvest, was the biomaterial implantation. The sterile fragments were incubated overnight at 37°C in Dulbecco's Modified

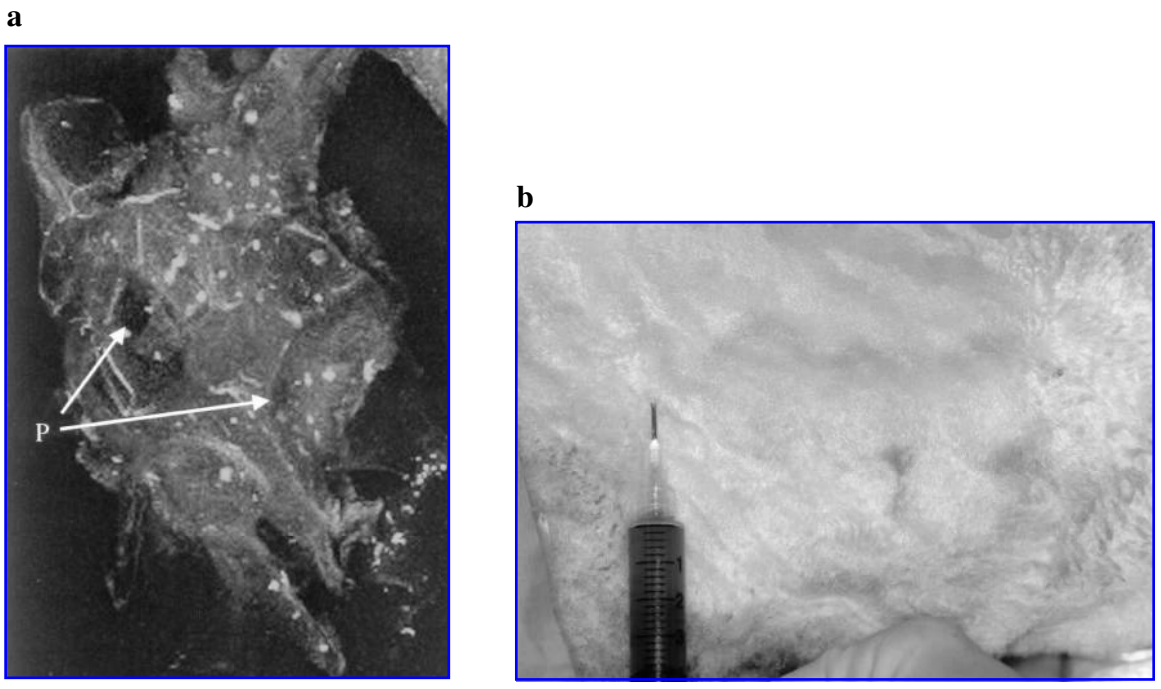

FIG. 1. **(a)** Laser scanning confocal microscope image of porous alginate fragments. Fragments were stained with eosin (5× magnification). **(b)** 2-mL injections of fragments into the nape of the neck of a sheep. Four injections were made on each side of the neck.

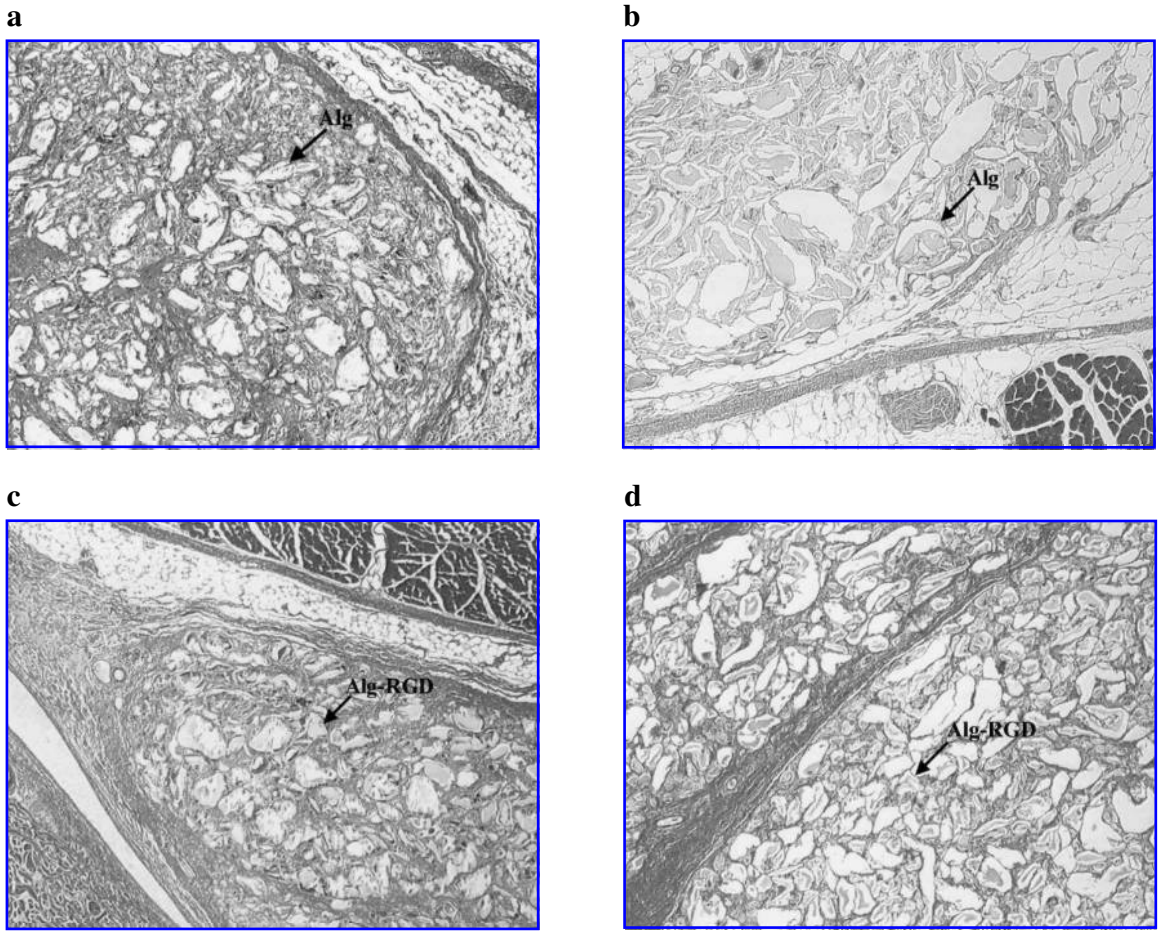

FIG. 2. Histological images (Masson's Trichrome stain) of alginate and alginate-RGD subcutaneous implants (50× magnification). Arrow indicates alginate material (alg). **(a)** 1-month alginate. **(b)** 3-month alginate. **(c)** 1-month alginate-RGD. **(d)** 3-month alginate-RGD.

Eagles Media (DMEMc; Fisher Scientific) containing 1% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO). Before the surgical procedure, the fragments were measured into 1-mL aliquots and then combined with 1 mL of DMEMc and loaded into a 5-mL syringe (Becton Dickinson and Company, Franklin Lakes, NJ). With the sheep under general anesthesia, a 2-mL total volume of material was injected into the nape of the neck with an 18-gauge needle (a maximum of four injections in each side of the neck; Fig. 1b). The widths of the implants were measured weekly using a GE LOGIQ 500 MR3 ultrasound with the H7039ML Linear LA39 probe (General Electric Medical, Milwaukee, WI) that emitted a frequency of 7.5 MHz.

Cell isolation, proliferation, and seeding onto biomaterials

Harvested omentum was placed into transport solution (TS) containing Hanks' balanced salt solution without calcium and without magnesium (Fisher Scientific) to which was added amphotericin B (10 μg/mL; Sigma-Aldrich) and penicillin-streptomycin (100 Units/mL and 100 μg/mL, respectively; Sigma-Aldrich). The tissue was rinsed three times with the TS, placed into 5 ml of TS, and minced with small scissors into 1- to 2-mm pieces. A maximum of 5 ml of minced tissue was transferred per 50-mL tube containing 20 mL of enzyme solution. The enzyme solution consisted of 4 mg/mL Collagenase I (Worthington Biochemical Corp., Lakewood, NJ) and 2 mg/mL bovine serum albumin (BSA, Sigma-Aldrich) in TS. The tube was agitated on its side at 37°C in 5% CO_2 on an orbital shaker at 85–95 rpm for 45 min or until approximately 1% of the original tissue was visible. Using a 5-mL syringe (Sherwood Medical, St. Louis, MO) fitted with a 15-gauge needle (Sherwood Medical), the enzyme mixture was triturated three to five times and subjected to centrifugation at 800 $\times$ *g* for 10 min. The fat and aqueous layers were aspirated, and the cell pellet was suspended in 20 ml of red blood cell lysis buffer (0.154 M NH_4CL, 10 mM $KHCO_2$, 0.1 mM EDTA, Sigma-Aldrich) and allowed to incubate for 10 min at room temperature. The suspension was then passed through a 40-μm nylon sieve (Fisher Scientific) and subjected to centrifugation at 800 $\times$ *g* for 10 min. The cells were washed once in 20 ml of TS and plated in DMEM (Fisher Scientific) supplemented with 10% FBS (Sigma-Aldrich), Insulin-Transferin-Selenium (0.01 μg/mL, 0.05 μg/mL, and 0.05 ng/mL, respectively; Sigma-Aldrich), 0.02 μg/mL FGF (Sigma-Aldrich), 2 mM L-glutamine (Life Technologies, Rockville, MD), 2.5 μg/mL amphotericin B (Sigma-Aldrich), and penicillin-streptomycin (100 Units/mL and 100 μg/mL, respectively; Sigma-Aldrich). The cells were then expanded in culture using the media described above. After five passages, the cells were seeded at a concentration of 3.0×10^6 cells/mL per 10 mL of alginate-RGD fragments in a roller bottle (850 cm^2; Fisher Scientific) that was pretreated overnight at 4°C with 3% BSA (Sigma-Aldrich) and then rinsed prior to use with sterile phosphate-buffered saline (Sigma-Aldrich). The roller bottle was rotated at 1.2 rpm, and the fragments were harvested 5 days postseeding. Cell viability was determined using fluorescent viability dyes of calcein AM and Ethidium homodimer–1 (Live/Dead™ kit from Molecular Probes, Inc., Eugene, OR), and three-dimensional images were captured using a Leica confocal microscope. Calcein AM emits a green fluorescence when cleaved by esterase in live cells (excitation of 495 nm and emission of 515 nm). Ethidium homodimer–1 emits a red fluorescence and only enters dead or dying cells (excitation of 495 nm and emission of 635 nm). Only four of six sheep yielded sufficient preadipocytes to seed onto biomaterials and inject into the subcutaneous space of the neck.

Tissue harvesting and histological processing

To evaluate all of the biomaterial implants, three animals were euthanized 1 month postinjection and three animals were euthanized 3 months postinjection. The implants were identified using ultrasound before euthanasia[23] and then harvested with a surrounding margin of connective tissue. The tissues were placed into Z-fix formalin (Anatech Ltd., Battle Creek, MI) and allowed to fix for a minimum of 48 h. The fixed tissue samples were cut in half at the mid-line to preserve the tissue biomaterial interface. These cut tissue blocks were processed using a paraffin embedding method. Four-micron sections were cut through the polymer-containing tissue perpendicular to the skin and muscle and stained with hematoxylin and eosin (H&E; Allegiance, McGraw Park, IL) and Masson's Trichrome (MT; PolyScientific, Bay Shore, NY) for the detection of collagen.

Data quantification

Histology slides for the injected alginate and alginate-RGD fragments were reviewed by two investigators and were scored using a scale of 0 for a minimal and 4 for a maximum response.[25] The slides were assessed for tissue ingrowth into the polymer substrate, vascular ingrowth into the implanted material, inflammatory response (giant cells, monocytes, and polymorphonuclear leukocytes [PMNs]), and capsule formation around the perimeter of the implant. Images of each slide were captured using an Olympus BX40 inverted microscope (Olympus Optical Incorporated, Japan) with a Sony PowerHAD 3CCD color video camera (DXC-950P, Sony, Inc., Japan) and the data processed using a Dell Dimension XPS R450 computer. The captured data were imported into Image Pro 4.0 (Media Cybernetics, Silver Spring, MD), and the width and area of the implants were measured.

Statistical analysis

Means and standard errors were calculated for all data, and an analysis of variance (ANOVA) was calculated using Scheffe's analysis and Statview, version 4.5® (Abacus, Berkeley, CA). Differences were deemed significant at a 95% confidence level ($p < 0.05$).

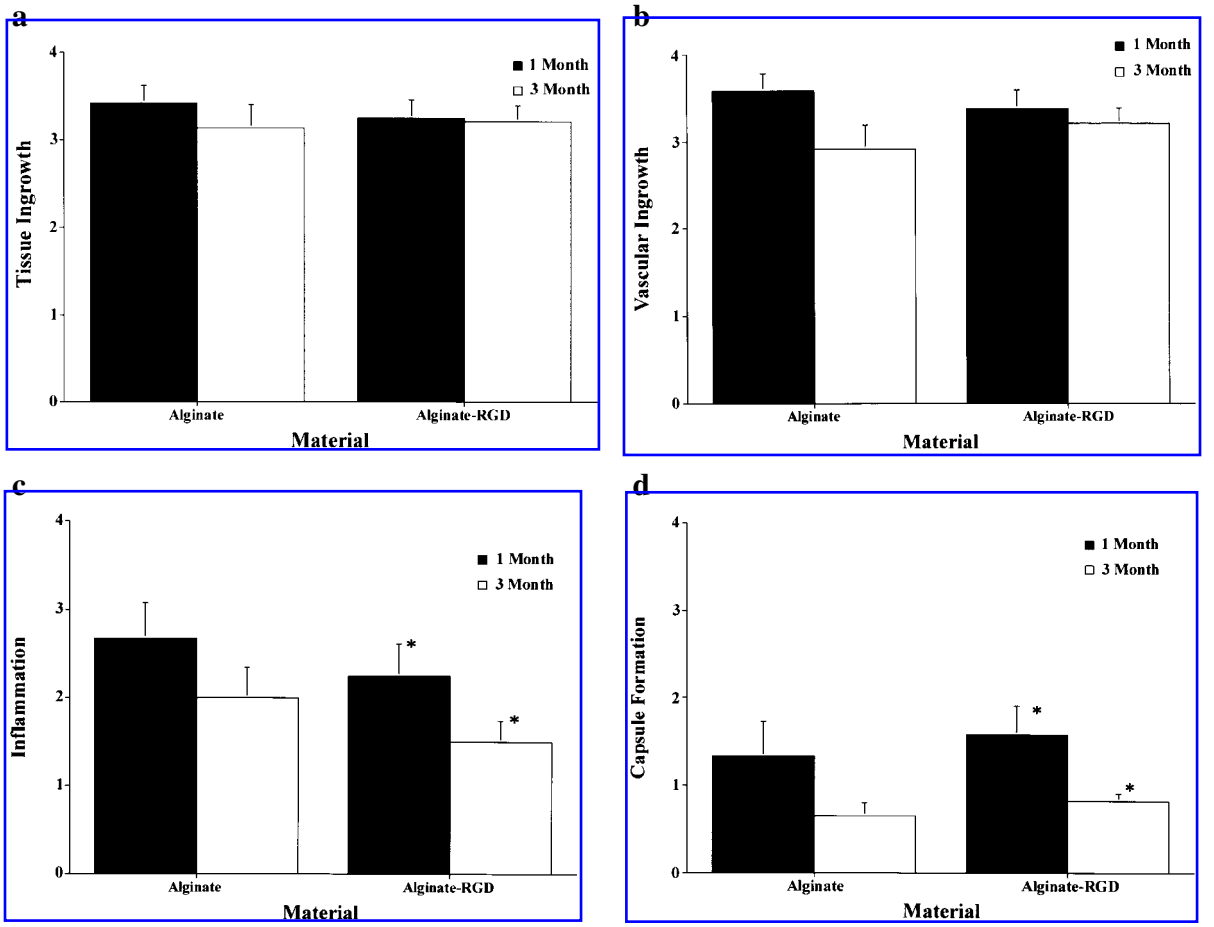

FIG. 3. Histological scoring of alginate and alginate-RGD implants at 1 and 3 months. **(a)** Tissue ingrowth. **(b)** Vascular ingrowth. **(c)** Inflammation. **(d)** Capsule formation around the implant ($n = 6$ for 1- and 3-month alginate and 1-month alginate-RGD; $n = 7$ for alginate-RGD 3-month implants). *Statistical significance $p < 0.05$.

RESULTS

The 2-mL injection of biomaterial plus DMEMc into the sheep's neck created a raised bleb within the subcutaneous space (Fig. 1b) that decreased in size by the fourth to fifth week due to the loss of fluid injected with the hydrogel fragments. It is unclear whether the fluid injected with the fragments had any bearing on tissue engraftment since all of the implants were injected using the same solution.

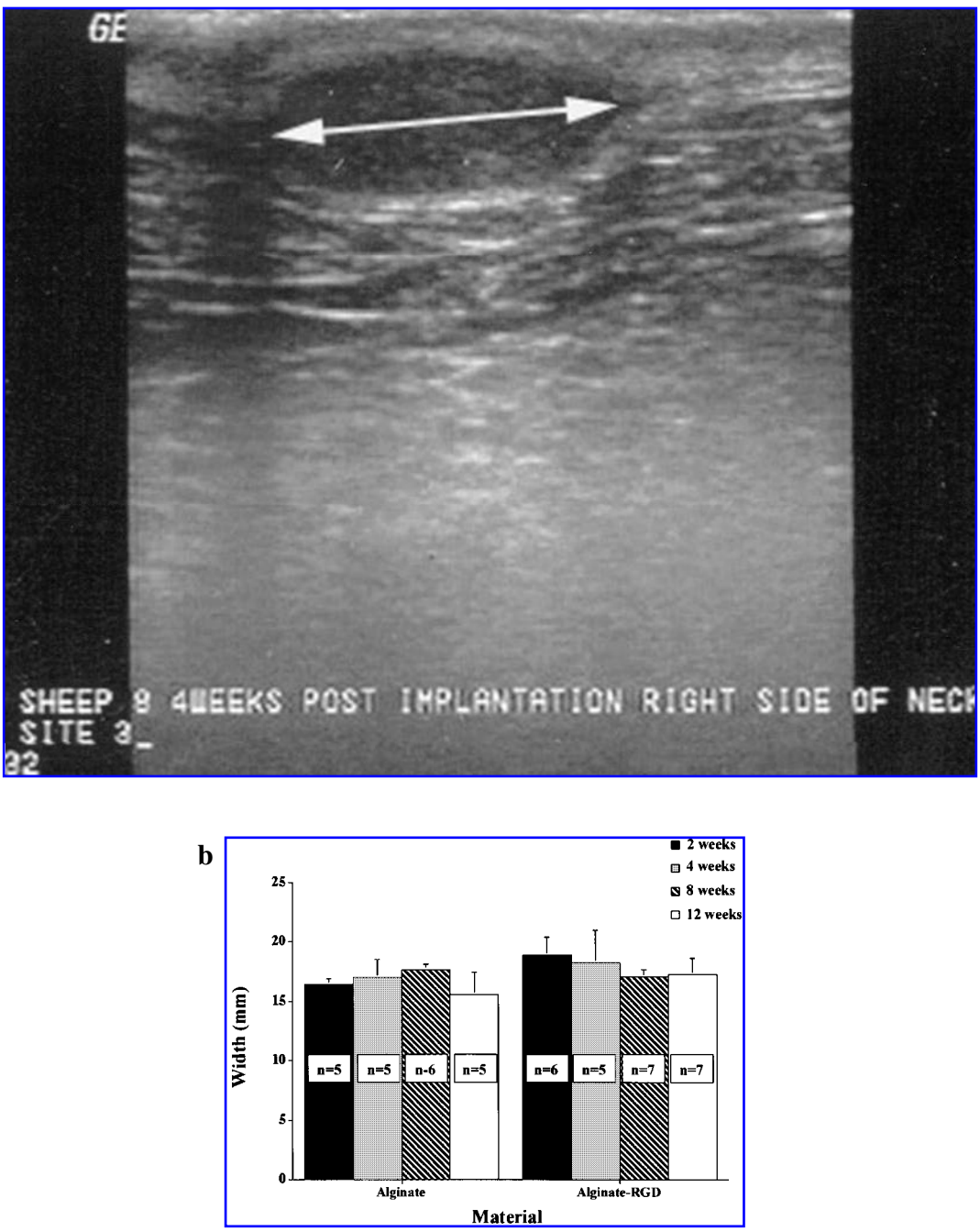

FIG. 4. **(a)** Ultrasound image of subcutaneous alginate fragment implant taken at 3 months. Arrow indicates width of implant. **(b)** Width (mm) of alginate and alginate-RGD implants measured using ultrasound over 12 weeks.

Representative histology slides for the alginate and alginate-RGD implants from 1 and 3 months are presented in Fig. 2a–d. Tissue and vascular ingrowth with a minimal capsule surrounding the materials is evident in all of the implant sites. The alginate and alginate-RGD are still present within the subcutaneous space at 3 months. Figure 3 presents the data generated from the scoring evaluation (0 = minimal and 4 = maximal). There was no statistical difference between the two materials in tissue ingrowth, and the ingrowth remained constant throughout the experiment (Fig. 3a). There was a well-established vascular bed in both materials (Fig. 3b), and there was no statistical change in vascular ingrowth over the time of the experiment. The inflammatory response, consisting mainly of giant cells and monocytes within the implant site, was more prominent at 1 month, and there was a statistically significant decrease between the 1- and 3-month time points for the alginate-RGD implant site (Fig. 3c). The inflammatory response was similar in magnitude for all of the conditions examined and could be due to the alginate, the DMEMc carrier solution, or a combination of both the hydrogel and the media. Weakly organized collagen surrounding the material implants was defined as the implant capsule and was noted to be minimal for both materials at 1 and 3 months (Fig. 3d). There was a significant decrease in the presence of a capsule for the alginate-RGD implants, with some grafts demonstrating complete integration with the surrounding tissue.

The width of the implants was examined weekly using high-frequency ultrasound.[27] Figure 4a is an ultrasound image of a 12-week alginate implant. The width of the implant was quantified using the ultrasound machine software. There was no statistical difference in implant width when comparing the two materials, and the width of the implants did not change during the experiment (Fig. 4b).

To confirm the ultrasound measurements, histology slides of both materials from the 1- and 3-month time points were measured for width and total area using an image analysis system. Again, there was no statistical difference in width or area for either material throughout the study (Fig. 5a,b). The widths measured on the histology slides were less than those measured by ultrasound, which could be due to the 15–30% shrinkage of the tissue that occurs during histological processing.[28,29]

Sheep preadipocytes isolated from the omentum, expanded in culture, and seeded onto the porous alginate-RGD fragments are shown in Figure 6. The sheep preadipocytes attached, proliferated, and spread onto the biomaterial surface. This material was subsequently implanted into the nape of the neck of some of the sheep to determine if new adipose tissue would form. Photomicrographs of histology slides of 1- and 3-month implantations are presented in Figure 7a–d. Well-defined adipose tissue was identified in the biomaterial transplant site. This was not observed in any of the acellular biomaterial implants. However, it is unknown whether the observed adipose tissue was due to the transplanted cells or new tissue ingrowth from the surrounding subcutaneous space.

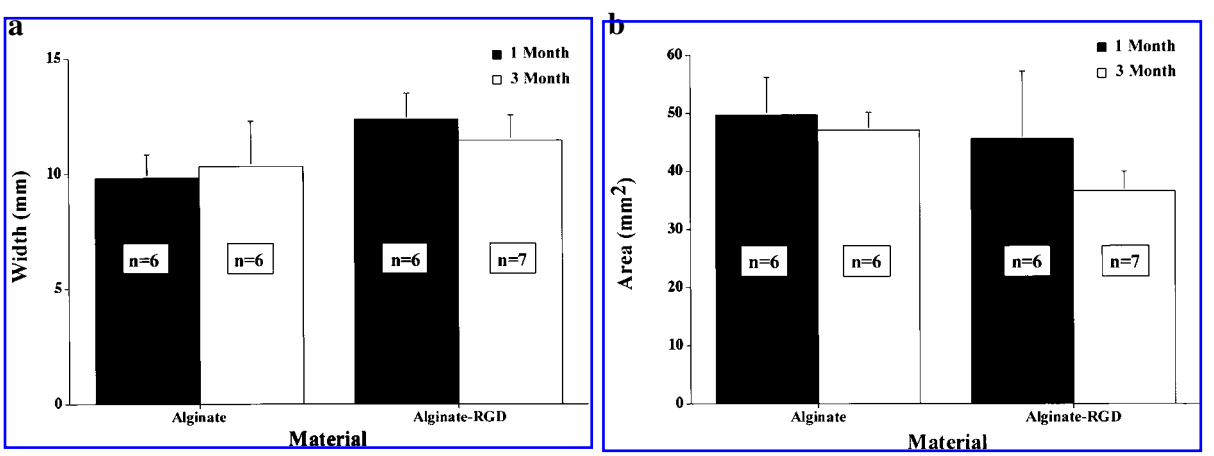

FIG. 5. **(a)** Width (mm) of alginate and alginate-RGD implants measured at 1 and 3 months on histology slides. **(b)** Area (mm^2) of alginate and alginate-RGD implants measured at 1 and 3 months on histology slides.

DISCUSSION

Both alginate and alginate-RGD injected into the subcutaneous space of the sheep's neck demonstrated minimal capsule formation, mild inflammatory response, and excellent tissue and vascular ingrowth throughout the interstices of the material. These are important observations because very few materials elicit a similar response in a subcutaneous implant site.

To be a useful material for a plastic and reconstructive construct, the implant must remain stable for an extended time. Our previous work with a rat subcutaneous implant model demonstrated that alginate was still present at 6 months, and the area of the implant did not change over this time.[25] To determine the width and presence of the implants over the course of the sheep experiment, we used high-frequency ultrasound.[27] High frequency ultrasound provided us the ability to monitor noninvasively the subcutaneous biomaterial implants over time. The implanted biomaterials are denser than the surrounding subcutaneous tissue. Hence, the emitted sound waves from the transducer reflect off of the biomaterial and are detected by the transducer that then depicts the implanted material as a shadow compared with the surrounding tissue. Both the ultrasound and corresponding histology measurements demonstrate that the material retains its shape and structure over the experiment.

To use this material for cell transplantation, the hydrogel structure must support cellular adhesion and growth. Previous work has demonstrated that this material supports murine cell adhesion and proliferation.[23,24,30] In the present study, we conducted initial experiments to isolate adipose tissue from the omentum of the sheep, expanded the cells, and seeded the pre-adipocytes onto the alginate-RGD porous fragments. The omentum was chosen as the adipose tissue source because of the lack of adequate subcutaneous adipose tissue. Using the omentum as the adipose tissue source, we had variable results in the isolation and expansion of preadipocytes, with two of six sheep yielding too few cells for a cell seeding and transplant experiment. This lack of a good subcutaneous adipose tissue supply hampers further use of the sheep for this study. Nevertheless, sites transplanted with preadipocyte/alginate-RGD constructs (Fig. 7a–d) appear to have adipose tissue closely associated with the material. This tissue structure was not observed in any of the alginate or alginate-RGD control implantation sites. Unfortunately, we cannot conclusively state that the adipose tissue observed is due to the transplanted cells. This is due to the implantation of autologous preadipocytes into an area that contains adipocytes. To solve this problem, a traceable marker, such as a transfected cell expressing green fluorescent protein or a fluorescent tracking dye, should be used. Additional work using a different large animal model must be performed to determine whether adipose tissue can be reproducibly developed within the transplant site.

CONCLUSION

This study examined the use of a naturally derived hydrogel material to engraft in the subcutaneous space of a sheep and incorporate with the surrounding host tissue. Both alginate and alginate-RGD elicited a mild inflammatory response and incorporated well with the surrounding host tissue. However, as we demonstrated here and previously,[23,30] alginate-RGD provided the advantage of promoting cellular adhesion and proliferation *ex vivo*. Hence, alginate-RGD provided the properties necessary for the success of constructs for soft tissue reconstruction.

FIG. 6. Confocal image of sheep preadipocytes seeded onto porous alginate-RGD fragments. Viable cells are stained with Calcein AM that emits a fluorescent green upon excitation (excitation of 495 nm and emission of 515 nm). Dead cells are stained with Ethidium homodimer–1 that emits a red fluorescence (excitation of 495 nm and emission of 635 nm). Magnification, 5×.

FIG. 7. **(a)** 1-month implantation of sheep preadipocyte seeded alginate-RGD fragments (40× magnification). **(b)** 1-month implantation of sheep preadipocyte seeded alginate-RGD fragments (200× magnification). **(c)** 3-month implantation of sheep preadipocyte seeded alginate-RGD fragments (40× magnification). **(d)** 3-month implantation of sheep preadipocyte seeded alginate-RGD fragments (200× magnification).

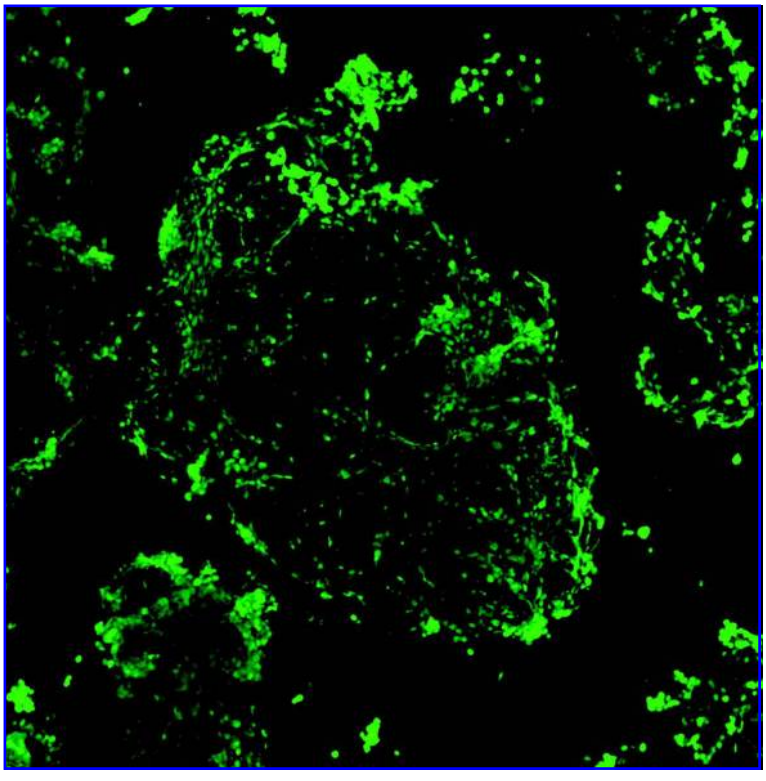

FIG. 6.

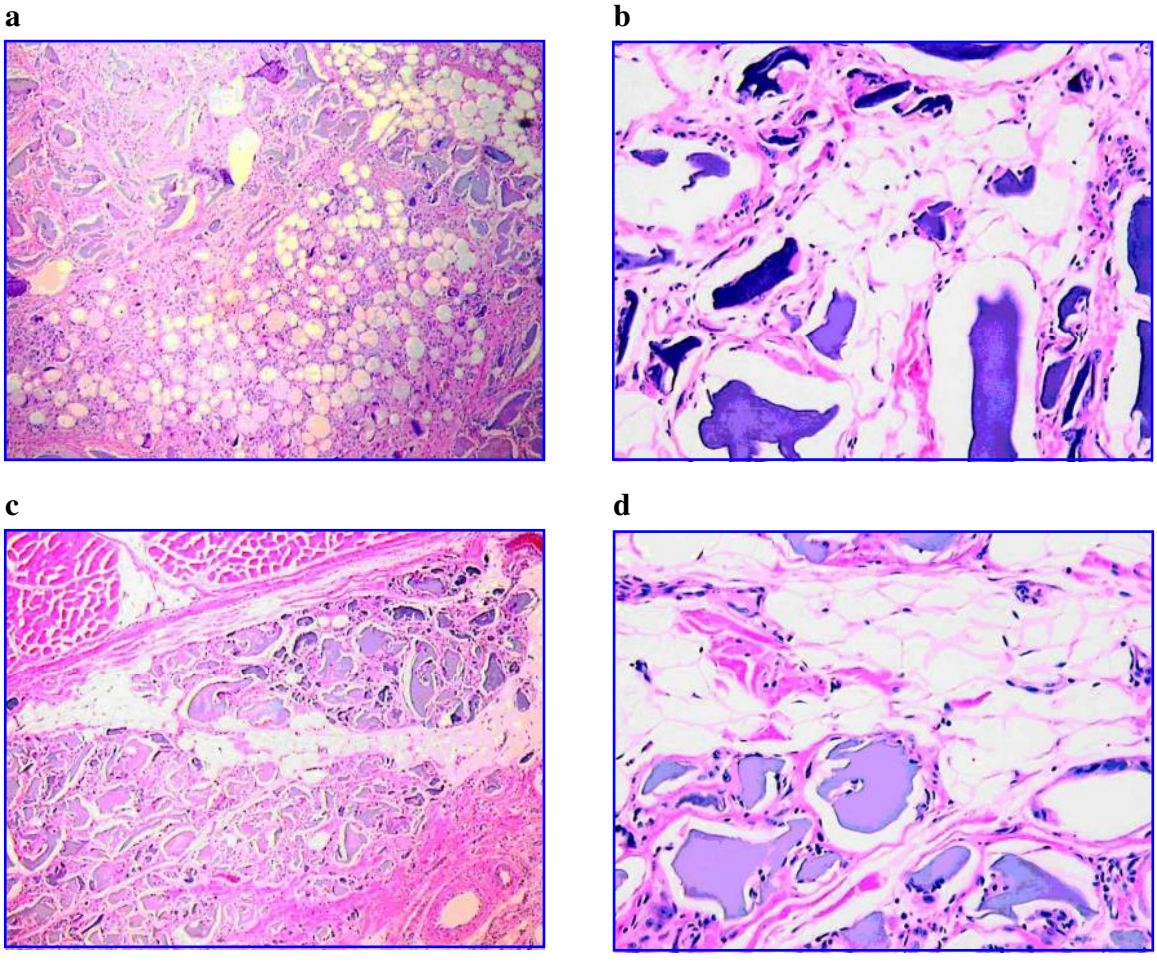

FIG. 7.

The long-term focus of our research is to develop subcutaneous tissue implants for the reconstruction of soft tissue defects. A specific goal is to develop an implant suitable for those who have had breast tissue removed (lumpectomy) for the treatment of breast cancer. Currently, there is no reconstructive option for these patients and therefore, many patients choose to have a mastectomy with total breast reconstruction. Our group is currently able to isolate, expand, and differentiate human preadipocytes from fat derived from a lipoaspirate. Our hope is that alginate-RGD in combination with autologous preadipocytes isolated from lipoaspirate can eliminate complex surgical procedures by providing an implant that will form new tissue and provide lumpectomy patients a reconstructive option.

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