Evaluation of the Safety and Efficacy of Periodontal Applications of a Living Tissue-Engineered Human Fibroblast-Derived Dermal Substitute. I. Comparison to the Gingival Autograft: A Randomized Controlled Pilot Study

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Background: Periodontists have found the gingival autograft to be an effective and predictable technique to increase the amount of attached gingiva around teeth, but this technique requires the surgeon to harvest donor tissue from a remote surgical site. The present study seeks to evaluate the safety and effectiveness of a tissue-engineered skin equivalent, a living human fibroblast-derived dermal substitute (HF-DDS), compared to a gingival autograft (GA) consisting of donor tissue harvested from the patient's palate in a procedure designed to increase the amount of keratinized tissue around teeth that do not require root coverage.

Methods: Twenty-five patients with insufficient attached gingiva associated with at least two teeth in contralateral quadrants of the same jaw were treated. One tooth in each patient was randomized to receive either a GA (control) or a HF-DDS graft (test). Clinical parameters measured at baseline and 3, 5, 7, 9, and 12 months included recession, clinical attachment level, keratinized tissue height, and plaque index. Probing depth was measured at 7, 9, and 12 months. Inflammation of each site was scored and texture and color of the grafted tissue were compared to the surrounding tissue. Resistance to muscle pull was evaluated and a questionnaire was used to determine patient preference. Surgical position of the graft and alveolar bone level were recorded at the surgical visit and patients were evaluated weekly for the first 4 weeks at which time recession and level of oral hygiene were measured. Biopsies and persistence studies were performed on a subset of the patients.

Results: Results for both test and control groups were similar for all measured clinical parameters with the exception of amount of keratinized tissue and percent shrinkage of keratinized tissue. The control group exhibited an average of 1.0 to 1.2 mm more keratinized tissue over time than the test group ($P < 0.001$) and the control group had about half as much shrinkage as the test group over time ($P < 0.001$). Test sites demonstrated significantly better color match over time compared to control sites. Similarly, tissue texture for test sites was significantly better than control sites over time.

Conclusions: Based on the results of this investigation, the tissue engineered HF-DDS graft was safe and capable of generating keratinized tissue without the morbidity and potential clinical difficulties associated with donor site surgery. The GA generated more keratinized tissue and shrank less than the HF-DDS graft, but the test graft generated tissue that appeared more natural. *J Periodontol 2005;76:867-880.*

KEY WORDS

Comparison studies; fibroblasts; gingival recession/surgery; gingival recession/therapy; grafts, dermal placement; grafts, gingival; grafts, keratinized tissue; tissue engineering.

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For many years periodontists have sought to develop therapies that would predictably increase the amount of attached gingiva around teeth. Denudation and pushback procedures were among the first techniques developed, but the outcome was unpredictable[1] and painful.[2] The lack of predictability was overcome with the advent of the gingival autograft (GA) in the 1960s.[3-6] Although effective, this technique does require a remote surgical site to harvest the donor tissue. This tissue is usually taken from the maxillary palatal region lingual to the bicuspids and molars. From the patient's perspective, the donor site is often more uncomfortable postoperatively than the graft site, and from the clinician's viewpoint, the donor site is more prone to postoperative problems such as excessive bleeding. In addition to these concerns, a finite amount of donor tissue is available to be harvested at any one time. For patients requiring multiple grafts, the amount of donor tissue available is insufficient to meet the patients' needs, and the patients are required to go through multiple surgical procedures, the surgeon harvesting the donor tissue, letting the palate heal, and then harvesting the tissue again. For these reasons, both the patient and clinician have been interested in an alternate source for donor tissue.

Sclera[7] and lyophilized dura matter[8,9] were used with little success as an alternate donor material for free gingival graft (FGG) in the 1970s. In the late 1970s and early 1980s, researchers' attention turned to freeze-dried skin (FDS) as a donor material. Although there were some favorable reports in the literature regarding its use,[10-12] the material never became widely accepted.

Cadaveric donor tissues resurfaced in the late 1990s, when acellular dermal matrix (ADM) was introduced to the dental profession as a source of donor material for soft tissue grafting. The periodontal literature regarding this material primarily centers around root coverage grafts, but there are limited numbers of reports on its use for augmenting keratinized tissue without root coverage.[13,14] Autogenous connective tissue has also been used to increase the amount of keratinized tissue around teeth where root coverage was not indicated.[15] Although the amount of donor tissue remains limited with this technique, the advantage is that the connective tissue is taken from a pouch in the palate, which some patients find less uncomfortable postoperatively than the GA.

A major goal of tissue engineering is the production of an unlimited supply of "off the shelf replacement parts" for the human body. Tissue engineered skin products have been used for treating burns, venous stasis, pressure and diabetic ulcers, and other maladies.[16-19] It is reasonable to assume that this technology could be harnessed for periodontics; Pini Prato et al.[20,21] reported on several cases where the patients' own fibroblasts were cultured and then implanted as donor tissue for gingival augmentation.

The purpose of this randomized, controlled within-patient paired design study is to evaluate the safety and effectiveness of a tissue-engineered skin product, a living human fibroblast-derived dermal substitute (HF-DDS), compared to a gingival autograft consisting of donor tissue harvested from the patient's palate in a procedure designed to increase the amount of keratinized tissue around teeth that do not require root coverage.

MATERIALS AND METHODS

Study Population

Twenty-five patients with insufficient attached gingiva (diagnosed by either increasing recession or a lack of keratinized tissue associated with chronic inflammation of the mucosa in the presence of good home care) adjacent to at least two teeth in contralateral quadrants of the same jaw who met the inclusion criteria were selected from patients seeking treatment in the author's (MKM) private practice from March 2000 to October 2001. All patients in the study were between 18 and 70 years old; willing and able to follow study procedures; had at least two non-adjacent teeth with an insufficient zone of attached gingiva that required soft tissue grafting; root coverage was not desired or indicated; and, if female and of child-bearing age, had a documented negative pregnancy test. Patients were excluded if they had any systemic conditions, i.e., diabetes, cancer, or HIV disorders that would compromise wound healing; chronic high-dose steroid therapy; bone metabolic diseases; or radiation or other immunosuppressive therapy that would preclude periodontal surgery. Demographics of the study population are presented in Table 1. A written Institutional Review Board-approved consent form regarding the study was obtained from each patient. The first three patients

Table 1.

Study Population (N = 25)

Age range	2.7 – 56.5	(mean 46.3 ± 8.18, median 49.2)
Gender		
Males	9	(36%)
Females	16	(64%)
Ethicnity		
Caucasian	22	(88%)
Hispanic	2	(8%)
Asian	1	(4%)
Current smokers	0	
Former smokers*	9	(36%)

* Mean years since smoking cessation 18.6; SD 6.8.

were used to determine surgical and material handling techniques and were not included in the statistical analysis. In case of adjacent teeth requiring grafting, only one tooth at each site was identified prior to surgery to act as the test or control tooth.

Clinical Assessment

The primary study objective was to determine if a human fibroblast-derived dermal substitute (HF-DDS) was capable of establishing a zone of keratinized tissue equivalent to the tissue generated facial to control teeth. The secondary end points included healing time, color and texture match of the grafted tissue to the adjacent tissue, resistance to oral muscle pull, probing depth, and patient preference.

During the patient screening, a medical history, complete dental history, and periodontal evaluation were performed. Preoperative documentation included the identification of the cemento-enamel junction (CEJ), the mucogingival junction (MGJ), and probing depth (PD) measured with an automated probe[‡] using a constant probing force of 25 grams with a 1 mm graded tip. The distance, measured to the nearest millimeter with a UNC 1.5 periodontal probe, from the free gingival margin to the mucogingival junction was recorded. At the outset, investigators used the visual method augmented by the roll technique when needed to identify the mucogingival junction. Mirrors inserted for photographic documentation stretch the tissues, causing difficulty in documentation of the MGJ. For that reason, a decision was made early in the study to also incorporate Schiller's iodine solution[22] to facilitate the detection of the MGJ. The alveolar mucosa stains dark brown because of high glycogen content, while the glycogen-free keratinized tissue lightly stains. Investigators determined the amount of attached gingiva by computing the distance from the free gingival margin to the mucogingival junction and then subtracting the probing depth. Dental radiographs were made of the study teeth and their preoperative clinical presentation was photographically documented at a standard magnification.

Patients were evaluated at weekly intervals for the first 4 weeks postoperatively, at which time any change in medications, adverse events, measurement of recession depth, level of oral hygiene, and postoperative instructions were recorded. Clinical photographs were also taken at these intervals. Following the 4-week visit, the patients were evaluated at months 3, 5, 7, 9, and 12. Any change in concomitant medications and/or adverse events was noted. Photographs were made of the test and control teeth. Inflammation of each site was scored, and texture and color of the grafted tissue was compared to the surrounding tissues.[23] Healing time was assessed. Healing was defined as the first point in time when the inflammation score was 0, indicating absence of any inflammation. Resistance to muscle pull (based on whether the free gingival margin of the tissue facial to the site moved when the adjacent cheek was retracted) was evaluated and a questionnaire was used to determine patient preference. The overall level of plaque control was recorded and oral hygiene instructions were reinforced as needed. Plaque score of the test and control teeth was recorded as presence or absence of plaque at the gingival margin and overall plaque index was evaluated using the modified O'Leary plaque index.[24] At each of these visits, both the position of the gingival margin as it related to a fixed reference point on the tooth, and the position of the MGJ was charted. Probing depth was recorded at the 7-, 9-, and 12-month visits. Three patients volunteered at 6 months to allow biopsies to be taken from the test and control graft for histological evaluation and comparison of the grafted tissue. Punch biopsies were taken of the test grafts on seven female volunteer patients at 3, 4, 6, or 18 months postoperatively to test for the presence of donor fibroblasts contained in the test graft. Training and calibration was conducted prior to the start of the study to ensure intraexaminer reproducibility with respect to outcome variables. The operator recorded at the time of surgery the alveolar bone level and the immediate post-surgical position of the gingival margin of the test and control graft. All postoperative evaluations were performed by the research coordinators, who were calibrated prior to the study and masked to the surgical procedures performed. Color, texture, inflammation, and resistance to muscle pull were scored independently by the two calibrated research coordinators.

Test Material

The tissue-engineered human dermal replacement graft[§] used in this study was manufactured through a three-dimensional cultivation of human diploid fibroblast cells on a polymer scaffold (Fig. 1). The scaffold is a bioabsorbable polyglactin mesh,[‖] which degrades by hydrolysis and is lost after transplantation, leaving the cellular and extracellular matrix components. The fibroblasts secrete a mixture of growth factors and matrix proteins to create a living dermal structure[17] which, following cryopreservation, remains metabolically active after being implanted on the graft bed. The human fibroblast cell strains used to produce this material come from newborn foreskins and are cultured by standard methods. The dermal implant contains normal matrix proteins, which play an integral role in providing structure as well as enhancing cell growth. The replacement graft also contains all of the glycosaminoglycans (GAGs) formed in young healthy dermis necessary for cell migration and binding growth factors. The fibroblasts remain metabolically active after

‡ Florida Probe Corporation, Gainesville, FL.
§ Dermagraft, Advanced Tissue Sciences, Inc., La Jolla, CA.
‖ Vicryl, Ethicon, Inc., Somerville, NJ.

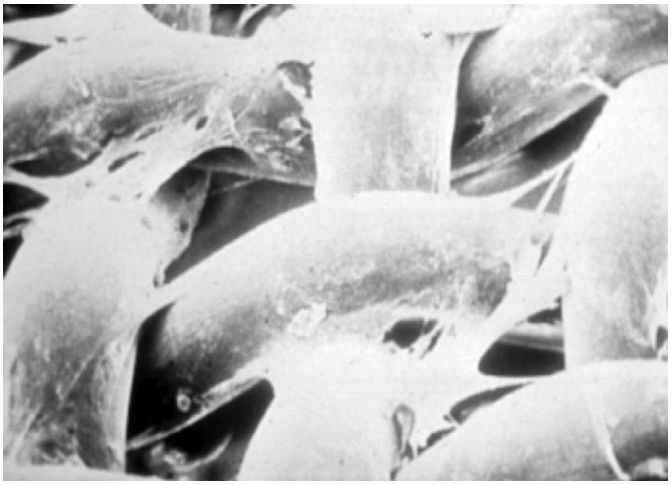

Figure 1.
Electron microscopy of polyglactin mesh with fibroblasts stretching across the spaces of the scaffold 1 to 2 days after seeding.

implantation and deliver growth factors, key to neovascularization, cell migration and differentiation.[25] Unlike keratinocytes, which carry surface human leukocyte antigens (i.e., HLA-DR) that may cause allograft rejection phenomena, implantation of allogenic human fibroblasts does not stimulate an immune response.[18]

Surgical Procedure

After meeting the entry criteria, each patient was assigned an identification number based on order of enrollment into the study. A predetermined randomization scheme was contained in a sealed envelope and labeled with the patient identification number. Immediately prior to treatment of each site, the envelope was opened and the two study sites were assigned the test or control treatment.

Following local anesthesia, the beds for the test and control grafts were created as described by Sullivan and Atkins.[6]

Test Site Coverage

The HF-DDS was delivered to the clinic frozen on dry ice. It was rinsed and thawed following the manufacturer's instructions. Using scissors, the investigator cut a piece of the HF-DDS from the bioreactor with a length corresponding to the mesial-distal dimension of the graft bed. The width of the graft (apico-coronal) of both test and control tissues was held constant at 5 mm. Once trimmed to size, the HF-DDS was carefully removed from the bioreactor and sutured in place with a 5-0 gut suture into the papillary region on the mesial and distal of the grafted tooth. Gentle finger pressure was applied through moistened gauze for approximately 1 minute to the HF-DDS to ensure intimate adaptation between it and the bed. The lip or cheek adjacent to the graft was then placed under tension to make certain that the graft was free of movement during muscle traction, and a surgical dressing[¶] that had been previously tested to ensure it was compatible with the HF-DDS was applied over the graft.

Control Site Coverage

Following the preparation of the recipient bed, a measurement of the length corresponding to the mesial-distal dimension of the graft bed was made. This measurement was carried to the premolar/molar region of the palate on the same side of the mouth as the control site. The investigator used a partial thickness (approximately 1 to 2 mm deep) incision to harvest a graft to the appropriate length and width (5 mm wide apico-coronal). The palatal donor tissue was secured to the recipient bed in an identical fashion as previously described for the HF-DDS.

Post-Surgical Care

All subjects received instructions in proper oral hygiene measures. Patients were instructed not to brush their teeth near the surgical sites for 2 weeks, but to use chlorhexidene gluconate (0.12%) mouth rinse for 1 minute twice daily for the first 4 weeks. This rinse had been tested prior to the study to ensure compatibility with the HF-DDS. The test and control sites were left covered with the surgical dressing until it fell off on its own or was removed at the 7-day postoperative visit. Patients were instructed to avoid excessive muscle tractioning or trauma to the treated area for the first 4 weeks. At 14 days, the patients were instructed in a brushing technique that would create minimal apically directed trauma to the soft tissue of the treated tooth. At week 4, the patient was instructed to resume gentle tooth brushing. Patients were instructed to resume both interproximal cleaning and chewing gradually in the treated areas. After 6 months, the patients were instructed in normal tooth brushing. All patients were seen weekly for the first 4 weeks. At these visits, any adverse events were recorded, changes in concomitant medications were noted, recession measurements made, clinical photographs obtained and oral hygiene instructions reviewed. At months 3, 5, 7, 9, and 12 all patients were recalled for prophylaxis and all clinical measurements were recorded along with the information mentioned above.

Biopsies

Histological evaluation. Three patients volunteered to allow biopsy of the test and control sites at 6 months. Under local anesthesia, an excisional biopsy approximately 3 mm wide by 3 mm long was made of the tissue above the periostium. The biopsies were immediately placed in separate containers filled with 10% neutral buffered formalin. Specimens were processed and stained with routine Harris hematoxylin and eosin

¶ Coe Pack, GC America, Inc., Alsip, IL.

stain. The biopsy was submitted for histological evaluation to compare the tissue generated through the test and control grafts. The examiner was masked to the treatments received.

Persistence study. Seven female patients volunteered to allow a 2 mm punch biopsy to be made of the test site to determine if the Y-chromosome fibroblasts remained. Three patients were biopsied at 3 months, one at 4 months, two at 6 months, and one at 18 months.

Statistical Analysis

The primary efficacy variable was the absolute change in the amount of keratinized tissue. Secondary efficacy variables were the absolute change in recession depth, clinical attachment level, and probing depth.

Summary statistics were computed for baseline clinical parameters by group. Comparisons of baseline parameters between groups were made using a paired *t* test. Measures of attached gingiva, recession depth, clinical attachment level, and probing depth over time were compiled for each subject. To test for differences in these parameters over time between test and control groups, analysis of covariance (ANCOVA) was conducted with corresponding baseline values included as a covariate. This ANCOVA model allowed for within-patient variation, treatment, and baseline values of the variable under analysis. Because sites and sequence of treatment were randomized, these factors were not included in the model and should have had no impact on the analysis.

Other end points analyzed included inflammation score, healing time, color and texture, resistance to oral muscle pull, and patient preference. The inflammation score for each site was scored independently by two calibrated raters. The kappa coefficient of agreement was calculated to measure inter-rater reliability. Ordinal inflammation scores for the two examiners were averaged for each site at each time point, and Wilcoxon signed rank tests were used to compare these scores at each time postoperatively. Repeated measures ANCOVA for paired data were also used to compare mean inflammation scores over two time frames: inflammation scores at 1, 2, 3, and 4 weeks; and inflammation scores at 3, 5, 7, 9, and 12 months.

Healing time was defined as the first point in time where the inflammation score was 0, indicating absence of any inflammation. Healing time was compared using Wilcoxon signed rank test.

Color and texture were scored independently by two calibrated raters. The kappa coefficient of agreement was calculated to measure inter-rater reliability. Ordinal color and texture scores for the two examiners were averaged for each site at each time point, and Wilcoxon signed rank tests were used to compare these scores at each time postoperatively.

Resistance to muscle pull was observed and was based on whether the free gingival margin with the tissue facial to the site moved when the adjacent cheek was retracted. All control and test sites exhibited resistance to muscle pull.

Patient preference was assessed with a questionnaire. Subjects rated each site for pain, sensitivity, swelling, and satisfaction at 3, 5, 7, 9, and 12 months. Preferences were then analyzed at each time point using Wilcoxon signed rank test.

Sample Size Determination

Prior to the initiation of this study, power calculations at the 5% significance level indicated that 20 evaluable patients were needed to detect a difference of 1.0 mm in change in recession depth with over 95% power. The calculations were based on an assumed within patient variation (standard deviation, estimated from previous studies with similar inclusion/exclusion criteria) of 1.0 mm. The sample size was calculated based on a paired analysis of the data.

RESULTS

Summary statistics for baseline parameters were computed by group and are shown in Table 2. Differences

Table 2.

Summary of Baseline Clinical Parameters by Treatment Group (N = 22)

	Mean	SD	Median	Range	*P**
Probing depth					
Test	1.14	0.47	1.0	0-2	
Control	1.41	0.59	1.0	1-3	0.011
Recession					
Test	3.11	1.43	3.0	0-6	
Control	3.05	1.17	3.0	0-5	0.753
Clinical attachment level					
Test	4.34	1.71	4.0	0-7	
Control	4.41	1.33	4.0	1-7	0.810
Keratinized tissue height					
Test	1.46	0.91	2.0	0-3	
Control	1.34	0.97	1.0	0-3	0.590
Alveolar bone level					
Test	5.46	1.57	5.0	2-9	
Control	5.36	1.43	5.0	2-8	0.760
Surgical position†					
Test	2.77	1.41	3.0	0-6	
Control	2.93	1.51	3.0	0-6	0.556
Plaque index					
Test	0.14	0.28	0.0	0-1	
Control	0.19	0.34	0.0	0-1	0.493

* Paired *t* test.
† Measurement taken at surgery from the coronal border of the graft to the cemento-enamel junction.

in baseline clinical parameters were also conducted using paired *t* tests. The only significant difference in baseline clinical parameters that was found was for probing depth with the control group having a significantly deeper initial mean probing depth of 1.41 mm compared to the test group with an initial mean probing depth of 1.14 mm ($P = 0.011$).

Inter-rater reliability for gingival inflammation, tissue texture, and tissue color was determined using the kappa coefficient of agreement. There was significant agreement between the two raters for all three measures with overall agreement of 81.9% ($\kappa = 0.69$, $P < 0.001$), 76.4% ($\kappa = 0.50$, $P < 0.001$), and 85.5% ($\kappa = 0.54$, $P < 0.001$) for gingival inflammation, tissue color, and tissue texture, respectively.

Probing depth, recession, clinical attachment creep, keratinized tissue, percent shrinkage of keratinized tissue, and plaque index were evaluated using repeated measures ANCOVA with patient, treatment, and corresponding baseline values included in the model to test for differences between the two treatments over time (Table 3). Keratinized tissue and percent shrinkage of keratinized tissue were the only parameters exhibiting a significant difference, with the control group exhibiting an average of 1.0 to 1.2 mm more keratinized tissue ($P < 0.001$) as well as only about half as much shrinkage as the test group ($P < 0.001$).

Over the course of the study, the operator experimented with varying layers of the test material. Five test sites received one layer while the remaining 17 test sites received three or more layers (15 received three layers and two received four layers). All statistical analyses were repeated with the test sites divided according to the number of layers of test material used (one layer versus ≥ three [three+] layers). When the two test groups were compared to the control group, no significant differences were detected, and the inference remained the same. A separate set of statistical analyses were conducted to compare the two test groups (one layer versus three+ layers) alone. In repeated measures ANCOVA, no significant differences were detected between the two test groups. However, when comparisons were made using Mann-Whitney *U* test, the test group with three+ layers had significantly greater keratinized tissue and significantly less shrinkage at 9 months (0.011) and 12 months (0.035) compared to the one layer test grafts; similar differences were also

Table 3.

Clinical Parameters over Time by Treatment Group (N = 22)

	3 Months Mean (95% CI)	5 Months Mean (95% CI)	7 Months Mean (95% CI)	9 Months Mean (95% CI)	12 Months Mean (95% CI)	*P**
Probing depth						
Test	-	-	1.38 (1.18-1.57)	1.32 (1.13-1.50)	1.42 (1.21-1.63)	
Control			1.63 (1.43-1.82)	1.50 (1.32-1.69)	1.17 (0.96-1.38)	0.563
Recession						
Test	2.97 (2.74-3.21)	3.07 (2.89-3.25)	2.91 (2.64-3.18)	2.98 (2.74-3.21)	3.00 (2.79-3.20)	
Control	2.80 (2.57-3.03)	2.86 (2.68-3.04)	2.68 (2.41-2.95)	2.80 (2.56-3.04)	2.78 (2.57-2.98)	0.137
Clinical attachment						
Test	-	-	4.31 (3.95-4.66)	4.29 (4.01-4.59)	4.27 (3.98-4.56)	
Control			4.28 (3.93-4.64)	4.30 (4.01-4.58)	3.96 (3.67-4.25)	0.575
Clinical attachment creep†						
Test	-	-	1.48 (1.16-1.81)	1.47 (1.16-1.78)	1.44 (0.76-1.76)	
Control			1.40 (1.08-1.72)	1.42 (1.11-1.73)	1.08 (0.76-1.40)	0.407
Keratinized Tissue						
Test	2.72 (2.45-2.99)	2.70 (2.39-3.01)	2.63 (2.32-2.94)	2.59 (2.31-2.86)	2.72 (2.42-3.03)	
Control	3.74 (3.46-4.01)	3.73 (3.43-4.04)	3.76 (3.45-4.07)	3.80 (3.52-4.08)	3.91 (3.61-4.22)	<0.001
% Shrinkage in keratinized tissue						
Test	45.5 (40.1-50.8)	45.9 (39.9-51.9)	47.3 (41.2-53.3)	48.2 (42.7-53.6)	45.5 (39.5-51.4)	
Control	25.5 (20.1-30.8)	25.5 (19.5-31.5)	25.0 (19.0-31.0)	24.1 (18.6-29.5)	21.8 (15.9-27.7)	<0.001
Plaque index						
Test	0.30 (0.23-0.36)	0.16 (0.11-0.22)	0.21 (0.12-0.29)	0.41 (0.32-0.50)	0.24 (0.20-0.27)	
Control	0.32 (0.26-0.38)	0.23 (0.17-0.28)	0.20 (0.12-0.29)	0.34 (0.25-0.43)	0.22 (0.19-0.25)	0.955

* For the overall difference between test and control groups based on repeated measures ANCOVA with patient, treatment, and corresponding baseline values included in each model.
† Based on the change in clinical attachment from the original surgical position.

Table 4.

Keratinized Tissue (mm) and Shrinkage (%) by Layers of Test Material

	N	Mean	SD	Median	Range	P*
Keratinized tissue						
3-month						
1 layer	5	2.40	0.55	2.0	(2-3)	
3+ layers	17	2.82	0.56	3.0	(2-4)	0.150
5-month						
1 layer	5	2.20	0.84	2.0	(1-3)	
3+ layers	17	2.85	0.49	3.0	(2-4)	0.074
7-month						
1 layer	5	2.00	1.00	2.0	(1-3)	
3+ layers	17	2.82	0.53	3.0	(2-4)	0.060
9-month						
1 layer	5	1.80	0.84	2.0	(1-3)	
3+ layers	17	2.82	0.53	3.0	(2-4)	0.011
12-month						
1 layer	5	2.00	0.94	1.5	(1-3)	
3+ layers	17	2.94	0.66	3.0	(2-4)	0.035
Shrinkage						
3-month						
1 layer	5	52.0	11.0	60	(40-60)	
3+ layers	17	43.5	11.2	40	(20-60)	0.150
5-month						
1 layer	5	56.0	16.7	60	(40-80)	
3+ layers	17	42.9	9.9	40	(20-60)	0.074
7-month						
1 layer	5	60.0	20.0	60	(40-80)	
3+ layers	17	43.5	10.6	40	(20-60)	0.060
9-month						
1 layer	5	64.0	16.7	60	(40-80)	
3+ layers	17	43.5	10.6	40	(20-60)	0.011
12-month						
1 layer	5	60.0	18.7	70	(40-80)	
3+ layers	17	41.2	13.2	40	(20-60)	0.035

* Mann-Whitney U tests.

Table 5.

Gingival Inflammation (%) Over First 4 Weeks (N = 22)

Gingival Inflammation	Week 1		Week 2		Week 3		Week 4	
	Test	Control	Test	Control	Test	Control	Test	Control
None	0.0	0.0	4.5	0.0	27.3	22.7	45.5	54.5
Partial mild	0.0	0.0	13.6	9.1	27.3	31.8	18.2	27.3
Complete mild	0.0	0.0	22.7	31.8	9.1	13.6	27.3	9.1
Moderate	0.0	15.0	45.5	45.5	36.4	27.3	9.1	9.1
Severe	100.0	85.0	13.6	13.6	0.0	4.5	0.0	0.0
P	0.102		0.485		0.747		0.148	

noted at 5 months and 7 months, although these differences failed to achieve statistical significance ($P = 0.074$ at 5 months and $P = 0.060$ at 7 months) (Table 4).

Frequency distributions of gingival inflammation by group for each of the first 4 weeks are shown in Table 5. Comparisons were conducted using Wilcoxon signed rank test. Repeated measures ANCOVA were conducted to test for overall differences in gingival inflammation over the first 4 weeks. Gingival inflammation declined significantly over the first 4 weeks ($P < 0.001$). Overall, there was no difference in gingival inflammation ($P = 0.698$) between test and control groups during this time period.

Gingival characteristics were also evaluated over time with gingival inflammation, tissue color, and tissue texture assessed by two independent raters. For compilation of frequency distributions of gingival inflammation, tissue color, and tissue texture, the least favorable evaluation for the two raters was included. However, for analysis of gingival inflammation, tissue color, and tissue texture, the mean ordinal value for the two examiners was computed for each site, and the Wilcoxon signed rank test was used at each time point to test for differences between test and control groups. Gingival bleeding (present or absent) at each time point was evaluated using a sign test. Frequency distributions for gingival bleeding, tissue color, tissue texture, and gingival inflammation with corresponding statistical tests at each time point are shown in Table 6. Test sites demonstrated significantly better color match compared to control sites. Similarly, tissue texture for test sites was significantly better than control sites. No significant differences between test and control groups in gingival bleeding were found at any time point. The only significant difference in gingival inflammation was found at 5 months, with the test group exhibiting less inflammation compared to the control group ($P = 0.034$). Repeated measures ANCOVA was conducted to test for overall differences in gingival inflammation over 3, 5, 7, 9, and 12 months. Gingival inflammation declined significantly ($P < 0.001$); however, there was no overall difference in gingival inflammation ($P = 0.883$) between test and control groups.

Time until healing was assessed as the first recall visit where no gingival inflammation was detected by at least one of the two examiners. The mean time until healing for the test group was 7.81 ± 6.35 weeks (median, 4 weeks; range, 2 to 20 weeks) and for the control group 6.86 ± 7.48 weeks (median, 4 weeks; range, 2 to 36 weeks). No significant difference in healing time was detected ($P = 0.546$) between the groups (Wilcoxon signed rank test).

Patient perceptions including assessment of pain, sensitivity, swelling, and satisfaction for each site treated were ascertained from a

Table 6.

Gingival Characteristics Over Time (N = 22)

	3 Months		5 Months		7 Months		9 Months		12 Months	
Characteristic	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
Gingival bleeding (%)										
No	100	100	100	100	90.9	100	95.5	90.9	95.5	95.5
Yes	0	0	0	0	9.1	0	4.5	9.1	4.5	4.5
*P**	1.000		1.000		0.500		1.000		1.000	
Tissue color (%)										
More red	31.8	27.3	4.6	13.6	0.0	4.6	4.6	0.0	0.0	4.6
Equally red	63.6	18.2	90.9	27.3	100.0	31.8	86.4	18.2	90.9	27.3
Less red	4.6	54.6	4.6	59.1	0.0	63.6	9.1	81.8	9.1	68.2
P†	0.012		0.004		0.001		< 0.001		< 0.001	
Tissue texture (%)										
Less firm	0.0	54.6	0.0	68.2	9.1	63.6	9.1	81.8	9.1	77.3
Equally firm	86.4	40.9	100.0	31.8	90.9	36.4	90.9	18.2	90.9	22.7
More firm	13.6	4.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
P†	0.002		< 0.001		0.001		< 0.001		0.001	
Inflammation (%)										
None	63.6	63.6	90.9	72.7	90.9	90.9	86.4	100.0	95.5	90.9
Partial mild	31.8	36.4	9.1	22.7	4.6	4.6	13.6	0.0	4.6	9.1
Complete mild	4.6	0.0	0.0	4.6	4.6	4.6	0.0	0.0	0.0	0.0
Moderate	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Severe	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
P†	0.623		0.034		1.000		0.102		0.564	

* Based on the Wilcoxon signed rank test.
† Based on Wilcoxon signed rank test; observations for the two raters were averaged for each site.

Table 7.

Pain, Sensitivity, and Swelling Over Time (N = 22)

	3 Months		5 Months		7 Months		9 Months		12 Months	
	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
Pain (%)										
None	13.6	13.6	13.6	13.6	13.6	13.6	13.6	13.6	13.6	13.6
Mild	50.0	54.6	54.6	59.1	54.6	59.1	54.6	59.1	50.0	59.1
Moderate	31.8	27.3	27.3	22.7	27.3	22.7	27.3	22.7	31.8	22.7
Severe	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6
*P**	0.564		0.564		0.564		0.564		0.157	
Sensitivity (%)										
None	63.6	59.1	63.6	59.1	63.6	59.1	63.6	59.1	63.6	59.1
Mild	31.8	31.8	31.8	31.8	31.8	31.8	31.8	31.8	31.8	31.8
Moderate	4.6	9.1	4.6	9.1	4.6	9.1	4.6	9.1	4.6	9.1
Severe	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
*P**	0.317		0.317		0.266		0.317		0.317	
Swelling (%)										
None	45.5	50.0	45.5	50.0	45.5	50.0	45.5	50.0	45.5	50.0
Mild	36.4	36.4	36.4	36.4	36.4	36.4	36.4	36.4	36.4	36.4
Moderate	13.6	9.1	13.6	9.1	13.6	9.1	13.6	9.1	13.6	9.1
Severe	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6
*P**	0.157		0.157		0.157		0.157		0.157	

* Based on Wilcoxon signed rank test.

questionnaire administered at 3, 5, 7, 9, and 12 months. Pain, sensitivity, and swelling were rated on a 4-point ordinal scale for each site and differences were assessed using the Wilcoxon signed rank test (Table 7); no significant differences were found. Subjects rated satisfaction on a continuous scale for each site (Table 8). Wilcoxon signed rank tests detected no significant difference in patient satisfaction.

DISCUSSION

Tissue engineering holds the promise of creating an almost unlimited supply of donor tissue. The most advanced area in tissue engineering is the manufacture of skin for treatment of patients with burns or chronic wounds.[16] The periodontal literature contains a few reports on surgeons using tissue engineering techniques to biopsy and then grow the patient's own cells to be used as donor tissue for GAs. Pini Prato et al.[20] reported on a patient in which a biopsy of the attached gingiva was taken from the opposite side of the mouth that required gingival augmentation. Fibroblasts were obtained from the biopsy and seeded onto a nonwoven 3-dimensional hydroxyapatite (HA) matrix (benzyl ester of hyaluronic acid). Ten days following the biopsy, the graft of cultured fibroblasts on the 3-dimensional resorbable membrane was sutured onto the periosteal bed. The paper stated the graft appeared epithelialized at 2 months, but it did not state how much keratinized tissue was generated. The photographs provided seemed to indicate that the increase was minimal. The case report also included a biopsy which demonstrated dense keratinized tissue and no signs of the membrane. The authors suggested that this technique of obtaining a large amount of donor

Table 8.

Patient Satisfaction Over Time (N = 22)

	Mean	SD	Median	Range	P*
3-month					
Test	9.35	1.75	9.45	(3.0-11.2)	
Control	9.29	1.35	9.20	(5.2-11.0)	0.418
5-month					
Test	9.73	1.30	9.95	(6.4-11.1)	
Control	9.71	1.49	10.10	(4.3-11.1)	0.900
7-month					
Test	9.92	1.37	10.10	(6.0-11.5)	
Control	9.92	1.36	10.20	(6.1-11.5)	0.775
9-month					
Test	10.14	1.12	10.40	(7.0-11.4)	
Control	10.12	1.06	10.55	(7.6-11.4)	0.391
12-month					
Test	9.91	1.54	10.20	(5.1-11.6)	
Control	10.20	1.13	10.35	(7.8-11.5)	0.231

* Based on Wilcoxon signed rank test.

tissue from a small biopsy was a potentially significant improvement over past techniques and greatly decreased postoperative patient discomfort.

Pini Prato et al.[21] published another case report in which they used the same technique mentioned above in an effort to generate keratinized tissue in six sites from five patients. In this study, small biopsies of epithelium and connective tissue were taken. The keratinocytes and fibroblasts were separated, and only the fibroblasts were cultivated, seeded onto the HA scaffold, and ultimately used as donor material and sutured onto the periosteal bed. At the conclusion of the study at 3 months, all treated sites showed an increase in the amount of fully keratinized tissue as demonstrated by histologic examination. The amount of keratinized tissue gained on the mid-buccal of the grafts was a mean of 1.5 mm (0.5 to 2.5) and an increase of keratinized gingiva of 1.5 to 2.5 mm. No information was given on how wide the initial graft was or how much shrinkage occurred. The paper reports that during the first 15 days, the grafts clinically appeared like granulation tissue with signs of neovascularization and after 30 days, the membrane was no longer detectable. At 3 months the grafted sites were epithelialized and tissue augmentation was obtained. Biopsy of the graft demonstrated dense keratinized tissue. The authors state that keratinocytes were not needed in the graft because keratinization of the gingival epithelium is controlled by morphogenic stimuli of the underlying connective tissue. They also state that the technique is limited to non-root coverage grafts because the cultured cells could not survive over avascular root surfaces.

In another study Momose et al.[26] used a similar technique to biopsy attached gingiva (epithelium and connective tissue) from the retromolar pad. The epithelial cells were seeded onto a collagen/silicone bilayer membrane and the cultures were expanded and then used as donor tissue for soft tissue grafts. This study reported that significant amounts of vascular endothelial growth factor (VEGF) and transforming growth factor–alpha, beta-1 (TGF-δ, β1) were released from the tissue engineered human gingival epithelial sheets. They postulated that this grafting material after implantation might have the potential to promote wound healing and tissue regeneration. The amount of keratinized tissue gained was not reported.

As illustrated above, some case reports on tissue-engineered substitutes for soft tissue grafting have been found. However, this paper represents the first controlled, randomized study to evaluate the safety and effectiveness of a living tissue-engineered human fibroblast-derived dermal substitute compared to the gingival autograft using donor tissue harvested from the patient's palate to increase the amount of keratinized tissue around teeth that did not require root coverage.

The mechanism of action of HF-DDS involves multiple components acting in concert, including colonization of the wound bed by cells, angiogenesis, and promotion of re-epithelization.[17] This is accomplished simultaneously along two fronts:[27] First, the dermal tissue fills the bed, producing a substrate that encourages keratinocyte migration to cover the graft with epithelium. The collagen and fibronectin provided by the HF-DDS are required for optimal keratinocyte attachment and migration. Second, the living fibroblasts make a variety of growth factors including angiogenic factors such as VEGF, matrix-stimulating factors such as TGF-β1, and keratinocyte stimulating factors such as keratinocyte growth factor (KGF).[16,19] These properties in combination act potentially in a synergistic manner to promote regeneration.[28] In the case of HF-DDS, the surface receptors of the fibroblasts are able to communicate with the native cells of the defect and modulate the secretion of growth factors, extracellular matrices and glycosaminoglycans to ensure that the exact amount needed is received and that secretion is modulated on an ongoing basis.[27]

The GAs used as a control in this study were approximately 1 mm thick, similar to a number of reports in the literature.[29,30] The thickness of the graft seems to influence its revascularization and shrinkage.[31] The HF-DDS is very thin (250 μm), which was easily supported initially by perfusion. Because the material is so much thinner than naturally occurring attached gingiva, a decision was made early in the study to vary the number of layers placed from one to four so that the effect of the number of layers on clinical appearance and shrinkage could be observed. When multiple layers were used, they were folded one upon another, the apico-coronal dimension

constant at 5 mm. Clinically, sites with multiple layers produced a more natural appearing attached gingiva than did those receiving one layer, and the results indicated that the former group had significantly greater keratinized tissue and significantly less shrinkage at 9 (0.011) and 12 months (0.035) compared to sites with one layer.

Although the thickness of the test and control grafts varied, the apico-coronal width of both grafts was 5 mm at the time of placement. Bowers[32] evaluated the facial attached gingiva width and found that it varied from 1 to 9 mm with the narrowest zone being in the mandibular cuspid/bicuspid region. Lang and Löe[33] found that a minimum of 2 mm of keratinized tissue was necessary for health. Based on this information, the initial width of 5 mm was considered sufficient as some shrinkage was expected as the graft matured. At 12 months the test sites exhibited 2.72 mm of keratinized tissue and the control sites exhibited 3.91 mm (1.0 to 1.2 mm more) ($P = <0.001$). The control group had about half as much shrinkage (21.8%) as the test group (45.5%) ($P < 0.001$). These results are comparable to the 30% to 45% shrinkage of GAs reported by Morman et al.[31] and the 47% shrinkage reported by Ward.[34] These were the only two parameters with significant differences between groups. The test sites demonstrated significantly better color match and tissue texture than the control sites, both of which are important patient-based esthetic outcomes.

Reports in the literature suggest that GAs may experience what is known as creeping attachment. Matter[35] found that creeping attachment occurred between 1 month and 1 year in his 5-year study of GAs. Bell et al.[36] found creeping attachment of 0.89 mm or 28% over 1 year. Our study found 1.44 mm creeping attachment for the HF-DDS and 1.08 mm for the GA over 1 year. The reason for the significantly greater amount of creeping attachment of the test graft is unknown.

The results indicated that there was no difference in any of the baseline parameters between test and control sites except for initial probing depth; however, this had no bearing on the outcomes since the tissue being probed at baseline was removed when creating the graft bed. There were no significant differences in probing depth between the two groups at the end of the study.

The concept of using a graft of fibroblasts without keratinocytes to create keratinized tissue is justified on a number of levels. Studies have demonstrated that the keratinization of gingival epithelium is controlled by the morphogenic stimulation of the adjacent connective tissue.[37,38] In addition, the HF-DDS produces a substrate of collagen and fibronectin that promotes keratinocyte migration over its surface. The fibroblasts also secrete keratinocyte growth factor and TGF-α, which is known to positively influence the growth of the keratinocyte layer.[39]

It has been postulated that only connective tissue from the gingiva and periodontal ligament have the ability to form keratinized epithelium.[40] In light of the results from this study, that concept must be reevaluated. Biopsies were performed of both the test and control sites on three patients 6 months following surgery. Two of the patients had one layer of HF-DDS and the third had three layers of HF-DDS. Histologically, all of the grafts appeared similar and no difference from a histological viewpoint could be seen between one and three layers. The biopsies of the GA and the HF-DDS histologically appeared similar: connective tissue covered by keratinized epithelium (Figs. 2 and 3). In all grafts, a zone of connective tissue was observed with normal appearing fibroblast cells. Histologically, the GA and the

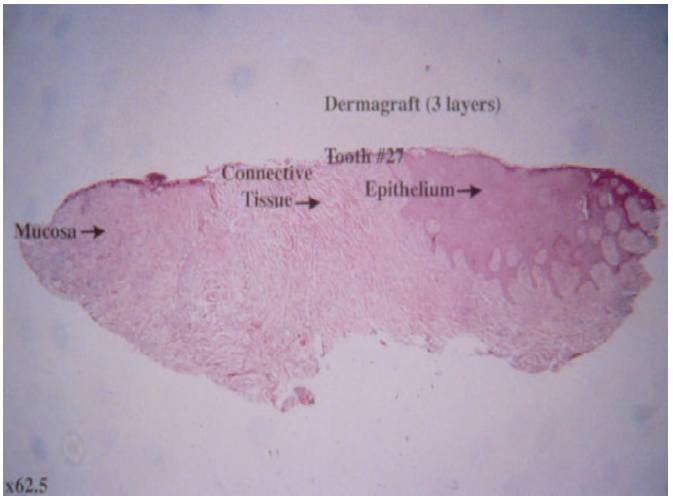

Figure 2.
Low-power view of HF-DDS graft at 6 months. Normal appearing connective tissue covered by epithelium.

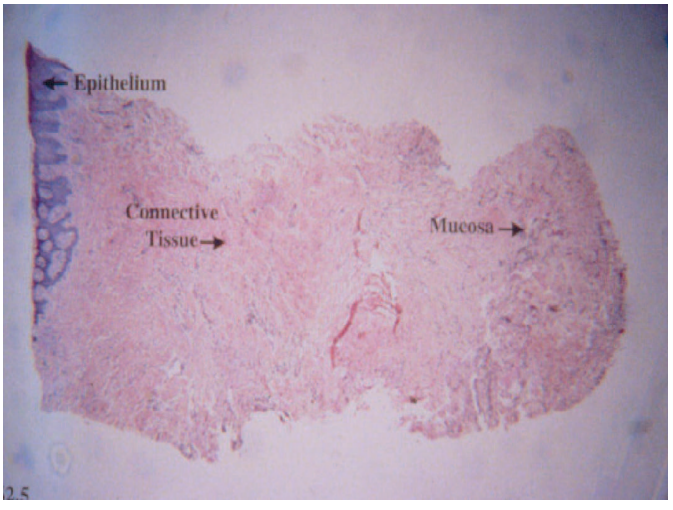

Figure 3.
Low-power view of GA at 6 months. Normal appearing connective tissue covered by epithelium.

HF-DDS appeared slightly different. The connective tissue in the GAs appeared histologically as "swirls" while the connective tissue of the HF-DDS appeared more organized. The significance of this, if any, is unknown although the organization of the cultured fibroblasts may have been affected by the manufacturing process. One must remember that the biopsy is taken only at one point in time, and as the tissue continues to remodel, the FGG and the HF-DDS may look even more similar.

In addition to the soft tissue biopsies, this study also evaluated 2 mm punch biopsies of the test graft in seven female patients, from 3 to 18 months after surgery to determine if any of the fibroblasts from the graft remained in the patient. The capacity of these fibroblasts to colonize the implantation site and survive was investigated by detection of the Y-chromosome marker SRY in the biopsy sample using a nested polymerase chain reaction (PCR) technique capable of detecting single molecules. Fibroblasts from the cultured implant were not detected in any patient in any biopsy. Furthermore, no adverse events were observed at any time. Because the first patients were biopsied at 3 months post-surgery, we do not know exactly when the fibroblasts disappeared. These data do seem to indicate the cells may disappear more quickly in the oral environment than in venous ulcers[28] where cells were detected for up to 6 months.

The test material has been used in studies for the treatment of burn patients, venous stasis ulcers, pressure ulcers and diabetic foot ulcers, with no adverse reactions definitely attributable to the material.

Oliver et al.[41] reported that GA epithelization is complete by 14 days and keratinization by 28 days. In the current study, the mesh was still apparent at the test sites at 7 days, but by 14 days the clinical maturation of the test and control grafts visually appeared to follow a similar course (Figs. 4 and 5). Overall, there was no difference in gingival inflammation or bleeding

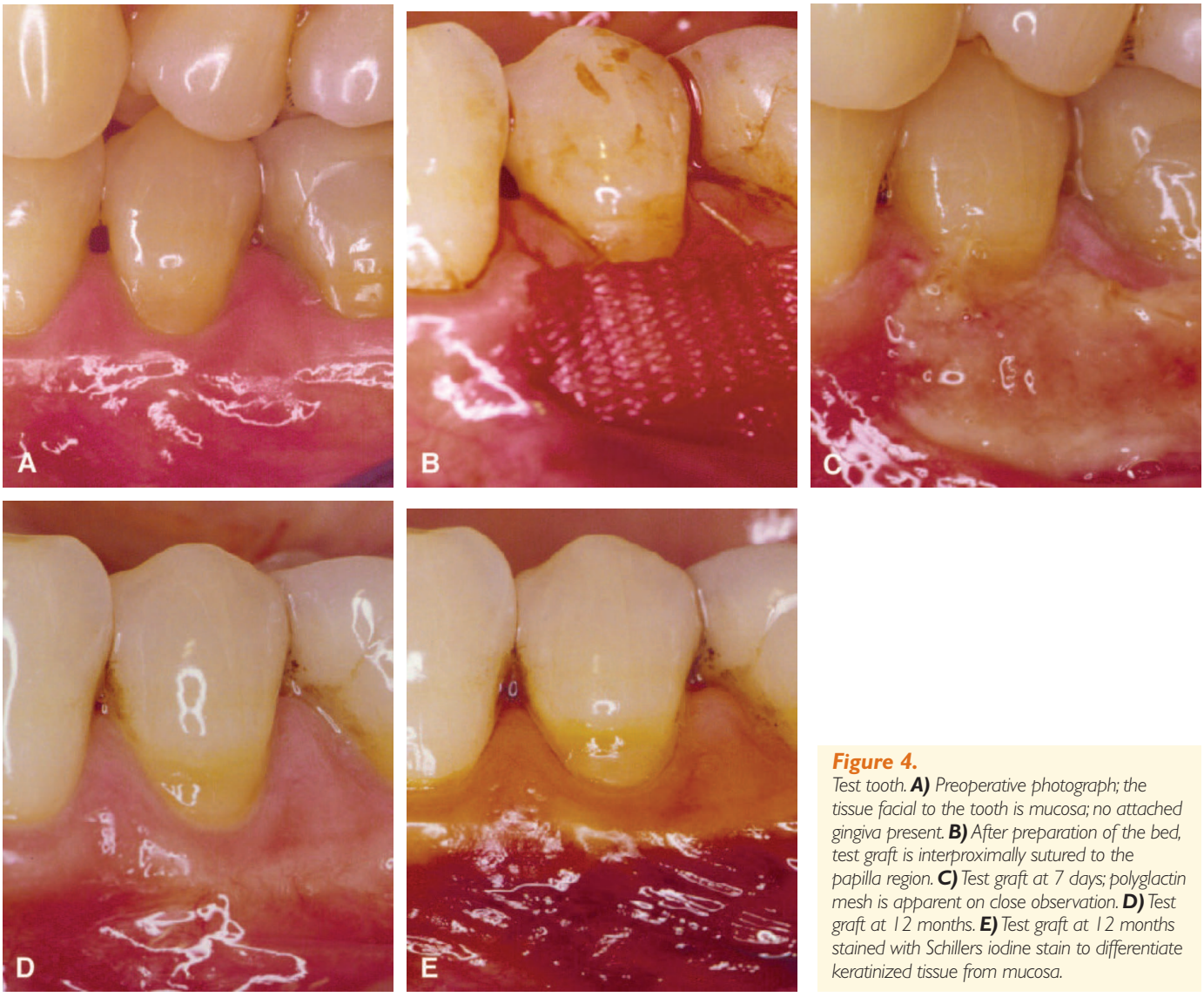

Figure 4.
Test tooth. ***A)*** *Preoperative photograph; the tissue facial to the tooth is mucosa; no attached gingiva present.* ***B)*** *After preparation of the bed, test graft is interproximally sutured to the papilla region.* ***C)*** *Test graft at 7 days; polyglactin mesh is apparent on close observation.* ***D)*** *Test graft at 12 months.* ***E)*** *Test graft at 12 months stained with Schillers iodine stain to differentiate keratinized tissue from mucosa.*

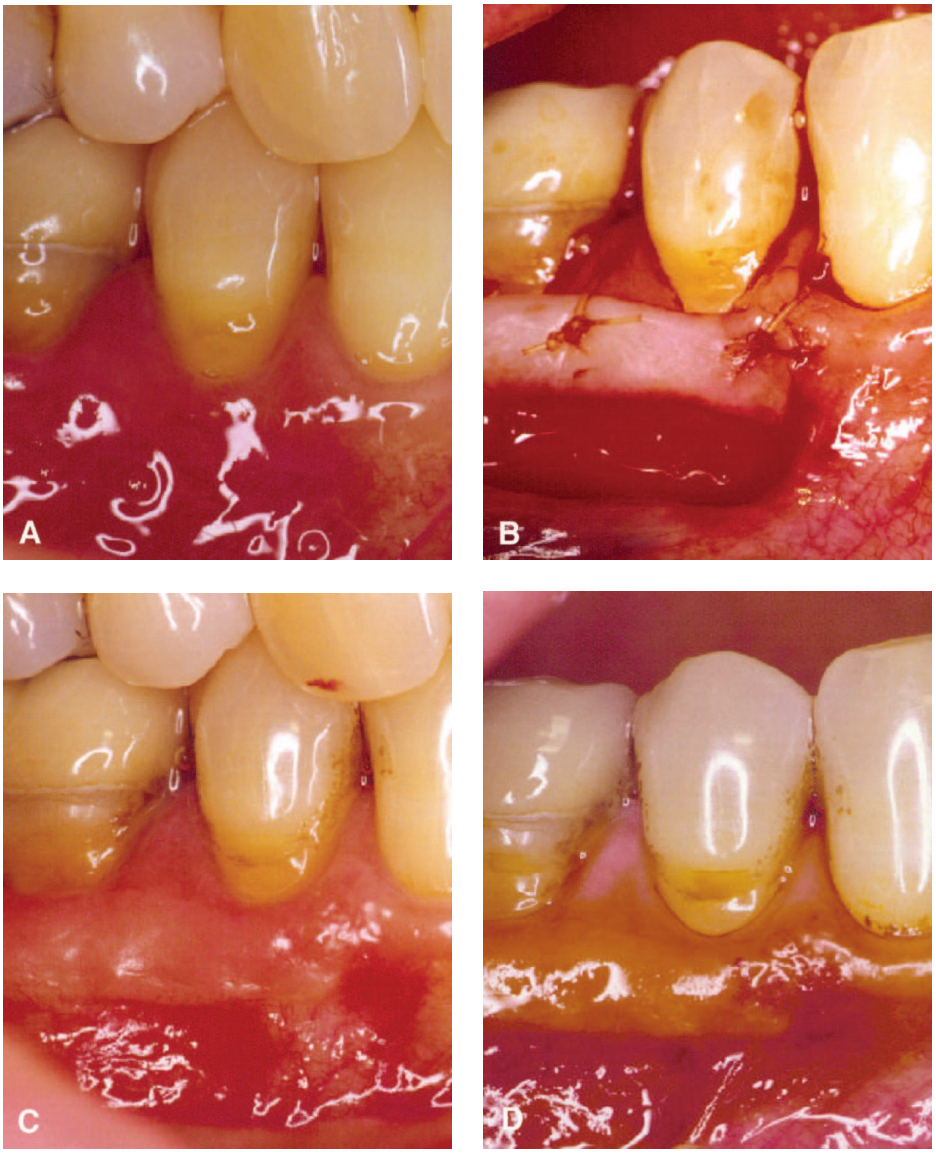

Figure 5.
Control tooth, same patient as Figure 4. ***A)*** *Preoperative photograph; the tissue facial to the tooth is mucosa; no attached gingiva present.* ***B)*** *After preparation of the bed, control graft is interproximally sutured to the papilla.* ***C)*** *Control graft at 12 months.* ***D)*** *Control graft at 12 months stained with Schillers iodine stain to differentiate keratinized tissue from mucosa.*

between the test and control groups and there was no significant difference in healing time.

Patient perceptions including assessment of pain, sensitivity, swelling, and satisfaction for each site treated were determined from questionnaires and no significant differences between the test and control groups were found. These results can be explained by the fact that the questionnaire was administered at 3, 5, 7, 9, and 12 months and asked the patient to compare the test and control site at that particular time. The study design was flawed in that it did not administer the questionnaire at weeks 1 through 4 and at the 1-month visit when important information on graft morbidity could have been collected. One of the obvious benefits of an alternative source of donor tissue should be less postoperative morbidity.

The results of this study clearly indicate that the HF-DDS graft shrank more and produced less keratinized tissue than the gingival autograft. Limited information exists in the literature regarding tissue engineered material's ability to create keratinized tissue around teeth. To the authors' knowledge, this information consists of three case reports.[18,19,41] The results of this study appear to be consistent with the observations in those case reports that, at the present time, it is possible to generate keratinized tissue with these tissue-engineered materials, but not in great quantities. This study represents the first attempt of using an "off the shelf" tissue-engineered material in the oral environment and, in retrospect, the investigator believes that to place the tissue-engineered material uncovered and exposed to the oral environment may have been a mistake. Better results may have been obtained if the test material had been covered by a flap, and this theory is the subject of Part II of this series.[42]

Many questions remain for future research. It is known that dermal fibroblasts behave differently than gingival fibroblasts or fibroblasts from the PDL.[43] What difference, if any, will this make when dermal replacement grafts are used as tissue replacement grafts in the oral environment? Many of the growth factors expressed by the HF-DDS have multiple functions. For example, polypeptide growth factors such as platelet-derived growth factor stimulate both cementogenesis and osteogenesis. What effects will these growth factors have on promoting regeneration of the attachment apparatus? Further studies are necessary to determine the type of attachment tissue that engineered grafts have to the root surface. The high unit cost of tissue-engineered products compared to traditional methods of treatment must also be reconciled.

CONCLUSION

The purpose of this randomized, controlled within-patient paired design, single-center study was to evaluate the safety and effectiveness of a tissue-engineered living human fibroblast-derived dermal substitute compared to a gingival autograft using donor tissue harvested from the patient's palate to increase the amount of keratinized tissue around teeth that did not require root coverage. The results demonstrated that, within the limits of this study, the tissue engineered graft is safe and capable of generating keratinized tissue. The amount and percent shrinkage of keratinized tissue were the only parameters that exhibited a significant difference between the test and control groups, with the control group exhibiting an average of 1.0 to 1.2 mm more keratinized tissue and about half as much shrinkage as the test group.

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