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### **AN AUTOLOGOUS CELL HYALURONIC ACID GRAFT TECHNIQUE FOR GINGIVAL AUGMENTATION: A CASE SERIES.**

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## An Autologous Cell Hyaluronic Acid Graft Technique for Gingival Augmentation: A Case Series

Giovan Paolo Pini Prato,\* Roberto Rotundo,\* Cristina Magnani,† Carlo Soranzo,† Leonardo Muzzi,\* and Francesco Cairo\*

**Background:** An autologous cell hyaluronic acid graft was used for gingival augmentation in mucogingival surgery.

**Methods:** Seven sites from 6 patients were used in this study. Five patients (5 sites) needed gingival augmentation prior to prosthetic rehabilitation, and one patient (2 sites) needed augmentation because of pain during daily toothbrushing. Full-mouth plaque score (FMPS), full-mouth bleeding score (FMBS), probing depth (PD), and clinical attachment level (CAL) were recorded for the sites at baseline and 3 months after surgery. The amount of keratinized tissue (KT) was measured in the mesial, middle, and distal sites of each involved tooth. A small 2 × 1 × 1 mm portion of gingiva (epithelium and connective tissue) was removed from each patient, placed in a nutritional medium, and sent to the laboratory. The gingival tissue was processed: keratinocytes and fibroblasts were separated and only fibroblasts were cultivated. They were cultured on a scaffold of fully esterified benzyl ester hyaluronic acid (HA) and returned to the periodontal office under sterile conditions. During the gingival augmentation procedure, the periosteum of the selected teeth was exposed, and the membrane containing cultivated fibroblasts was adapted to and positioned on the site.

**Results:** Three months after surgery, an increased amount of gingiva was obtained, and the histological examination revealed a fully keratinized tissue on all the treated sites.

**Conclusion:** Tissue engineering technology using an autologous cell hyaluronic acid graft was applied in gingival augmentation procedures and provides an increase of gingiva in a very short time without any discomfort for the patient. *J Periodontol* 2003;74:262-267.

### KEY WORDS

Gingiva/surgery; mouth mucosa/surgery; tissue engineering.

Regenerative medicine, therapeutic cloning, stem cell research, longevity research, and tissue engineering represent the most exciting arena for medical study and practice in the new millennium. Tissue engineering technology has already been developed and applied in different medical fields to replace cartilage, bone, cardiovascular components, the pancreas, and skin.<sup>1</sup> In dermatology, for example, the ability to produce a large amount of dermal-epidermal tissue from a small portion of the patient's skin in a short amount of time makes it possible to treat multiple lesions, such as extensive burns, diabetic ulcers, etc. In fact, when a large quantity of dermal-epidermal cells supported by a biological-resorbable scaffold is positioned over the exposed granulation tissue, favorable and rapid closure of skin wounds is possible, thus significantly improving the prognosis.<sup>2,3</sup> This therapeutic approach is based on the cultivation of human keratinocytes/fibroblasts in the laboratory, which are transferred to the patient on biological carriers.<sup>4-6</sup> One of the most widely used carrier supports is hyaluronic acid (HA). HA is a naturally occurring non-sulphated glycosaminoglycan consisting of a linear sequence of D-glucuronic acid and N-acetyl-D-glucosamine and is found in connective tissue, the synovial fluid of joints, and the vitreous humor of the eye. Furthermore, HA is involved in many biological processes such as tissue hydration, proteoglycan organization in the extracellular matrix, cell differentiation, cell behavior, and tissue repair. A fully esterified benzyl ester of HA exhibits the properties of highly biocompatible biopolymers. Recently, 3-dimensional scaffolds consisting of the benzyl ester of hyaluronan<sup>8</sup> have been used to support keratinocyte and fibroblast growth<sup>7,8</sup> (Figs. 1 and 2). The HA membrane is completely resorbed in the wound area within 4 weeks. Neovascularization and reinnervation in the cultured keratodermal graft are almost complete at 6 weeks.<sup>9</sup>

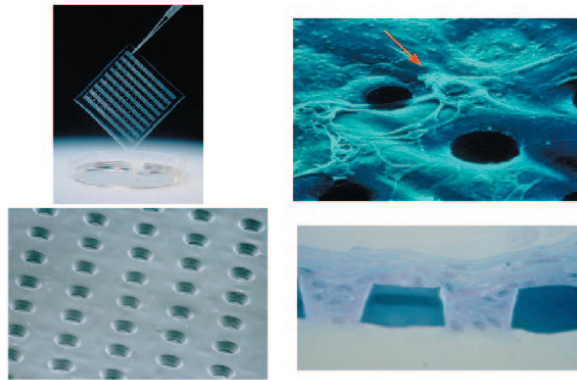
Based on this knowledge, tissue engineering technology may also be applied in periodontology. The

\* School of Dentistry, University of Florence, Florence, Italy.

† Private practice, Florence, Italy.

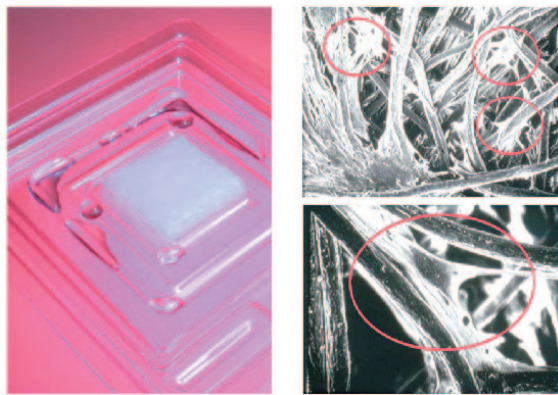
‡ Fidia Advanced Biopolymers srl, Abano Terme (Padova), Italy.

§ HYAFF, TISSUEtech Laboratory, Fidia Advance Biopolymers srl, Abano Terme (Padova), Italy.



**Figure 1.**

*In vitro* cultivation of keratinocytes onto a hyaluronic acid membrane (HA). Notice the keratinocytes (red arrow) passing through the holes of the resorbable HA support.



**Figure 2.**

*In vitro* cultivation of fibroblasts onto an HA membrane. Notice several fibroblasts (red circles) (scanning electron microscopy; original magnification  $\times 310$ ) and one fibroblast (SEM, original magnification  $\times 1,250$ ) on the membrane.

surgically denuded periosteum scheduled for a gingival augmentation technique in mucogingival procedures is a condition similar to dermatological wound exposure. In this case, the exposed recipient site, which is an ideal blood supply source, can be covered with cultivated keratodermal cells, instead of a traditional epithelial-connective tissue graft. A recently published clinical case report<sup>10</sup> has demonstrated an increased amount of keratinized tissue obtained from a small portion of the patient's gingiva in a short period of time.

The aim of this study is to demonstrate how the technique can be applied to periodontology and to highlight the advantages of tissue engineering technology in gingival augmentation procedures.

## MATERIALS AND METHODS

### Study Population

Seven sites from 6 patients were used in this study. Six patients, 2 males and 4 females aged 24 to 38, who needed to undergo gingival augmentation procedures were admitted to a private practice. The patients agreed to participate in the study and signed the consent forms in compliance with the Helsinki Declaration on Human Experimentation. All of the patients showed a reduced amount of gingiva: 5 patients needed to undergo prosthetic rehabilitation (patients #1, 2, 3, 4, 5), while one patient (#6) complained of discomfort during oral hygiene practices (Table 1).

### Measurements

Full-mouth plaque score (FMPS), full-mouth bleeding score (FMBS), probing depth (PD), and clinical attachment level (CAL) were recorded for the involved teeth at baseline and 3 months after surgery. Identification of the mucogingival junction was facilitated by staining with Schiller's IKI solution. The amount of keratinized tissue (KT = distance between the gingival margin and mucogingival junction) was measured in the mesial, middle, and distal sites for each involved tooth (Table 1 and Fig. 3).

### Procedure

The actual procedure consisted of 4 different phases: 1) gingival biopsy; 2) in-laboratory cell cultivation; 3) surgical procedure for application of the cultivated cells; and 4) postsurgical phase and follow-up.

**Gingival biopsy.** Under local anesthesia, a small portion ( $2 \times 1 \times 1$  mm) of gingiva (epithelium and connective tissue) was removed from the gingiva of the alveolar process using a surgical blade (Fig. 4). Slight pressure was applied to the donor site, which was left without suturing. The gingival tissue was placed in a nutritional medium (Dulbecco's modified Eagle medium, DMEM) containing 5% fetal calf serum (FCS) and antibiotics (penicillin 100 IU/ml, streptomycin 100  $\mu$ g/ml) and taken to the laboratory. The patients were instructed to use chlorhexidine digluconate 0.12% mouthwash for several days. They were told that they would be called in for surgery as soon as the cultivated cells arrived from the laboratory.

**In-laboratory cell cultivation.** In accordance with the laboratory's technological procedures,<sup>11</sup> the gingival tissue underwent the following process. Upon arrival at the laboratory, the tissue was rinsed in phos-

|| TISSUEtech Laboratory, Fidia Advanced Biopolymers srl.

**Table 1.**  
**Baseline Measurements of Treated Sites**

Patients	Baseline							
	Gender	Age	Tooth	PD (mm)	CAL (mm)	KT (mm)		
						Mesial	Middle	Distal
1	F	38	#22	1.0	1.0	2.0	1.5	2.0
2	M	34	#27	1.0	1.0	1.5	0.5	1.0
3	F	24	#20	1.0	5.0	2.0	0.5	2.0
4	F	38	#27	1.0	1.5	1.5	1.0	1.5
5	M	32	#3	2.0	3.5	2.0	1.0	1.5
6	F	35	#24	0.5	4.0	1.0	1.0	1.5
			#25	0.5	4.0	1.0	1.0	1.0



**Figure 3.**  
The apico-coronal extension of keratinized tissue is measured at the mesial, middle, and distal point of each involved tooth.



**Figure 4.**  
A biopsy (2 × 1 × 1 mm) is harvested from the gingiva.

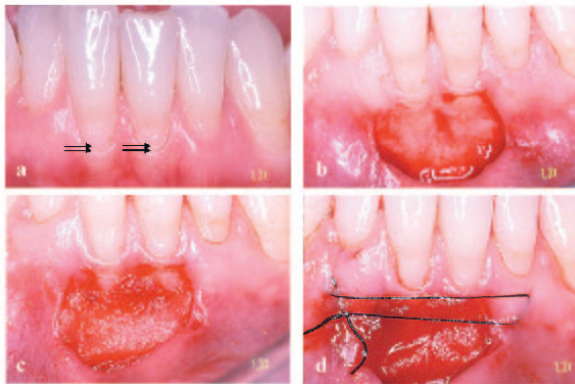
phate buffered saline (PBS) and transferred to a Petri dish containing 10 ml of dispase (5 mg/ml). After incubation at 37°C for 1 hour, the epidermal layer was gently peeled off from the dermis and rinsed with PBS. Human fibroblasts were obtained by overnight digestion of the deepidermalized dermis with a solution of 80 U/ml of type 1 collagenase<sup>¶</sup> at 37°C, 5% CO<sub>2</sub>. Cells were propagated in DMEM containing 10% FCS; the culture medium was renewed twice a week.

Cells were trypsinized at 80% confluence and split 1:3 for subsequent phases. Fibroblasts were cultured onto the non-woven matrix of benzyl ester of hyaluronic acid at a density of 8.0 to 10.0 × 10<sup>4</sup> cells/cm<sup>2</sup>, in DMEM containing 10% FCS; the culture medium was changed twice a week. On the eighth day after fibroblast seeding, a 2 × 4 × 0.1 cm 3-dimensional HA scaffold containing the patient's cultured fibroblasts was rinsed in PBS several times to remove the FCS. Cell construct was carried out by an ISO 9002 Certified Service<sup>#</sup> in a dedicated facility. Special care was applied to reagents used in the process: all reagents were supplied by certified companies. Concerning FCS, the product is certified by the European Pharmacopea as TSE-free, according to European requirements.

Fibroblasts cultured onto the scaffold were transferred to a sterile plastic tray filled with nutritional serum-free medium, sealed, and double-packaged under sterile conditions. The material was returned to the periodontal office 15 days after the biopsy.

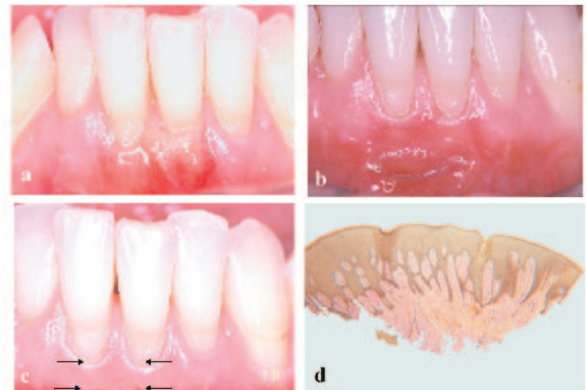
**Surgical procedure.** The 2 × 4 × 0.1 cm graft had to be applied within 24 hours of receipt at the periodontist's office. The recipient site (Fig. 5a) was prepared under local anesthesia. A partial-thickness flap was raised, and the underlying periosteum was exposed (Fig. 5b). The non-woven 3-dimensional membrane carrying the patient's fibroblasts was then removed from the sterile package, shaped, and adapted onto the recipient site (Fig. 5c). The membrane in the recipient site was stabilized with single sutures (Fig. 5d). No surgical dressing was applied.

¶ Whorington Biochemical Corp., Lakewood, NJ.  
# TISSUEtech Autograft System, Fidia Advanced Biopolymers srl.



**Figure 5.**

Case #6. **a)** The extremely reduced amount of gingiva (arrows) near the lower central incisors causes discomfort for the patient during daily oral hygiene. **b)** The exposed periosteum. **c)** The membrane containing the patient's cultivated fibroblasts is positioned onto the recipient site. **d)** The membrane is fixed with a single silk suture.



**Figure 6.**

Case #6. **a)** Fifteen days later, the granulation tissue is still present in the healing area, and the HA membrane is completely resorbed. **b)** One month later, the treated area is epithelialized, but keratinization is not complete. **c)** Healing after 3 months; the apico-coronal amount of gingiva (arrows) is increased. **d)** Histological examination shows full keratinization of the newly formed tissue after 3 months (van Gieson's stain; original magnification  $\times 60$ ).

**Postsurgical phase and follow-up.** The healing phase was completely free of pain. During the first 2 weeks, toothbrushing was discontinued and no mouthwash rinses were prescribed in order to avoid damage to the fibroblasts. A soft diet was also prescribed. After this phase, supragingival plaque control was restarted. During the first 15 days, a granulation-like tissue appeared, showing signs of neovascularization (Fig. 6a). After 30 days, the membrane was no longer detectable and had been replaced with newly formed tissue (Fig. 6b). Three months later, the grafted site appeared epithelialized, and tissue augmentation was obtained (Fig. 6c). At this time, histological examination showed the presence of dense keratinized tissue (Fig. 6d).

## RESULTS

The procedure was painless, and all patients were completely satisfied. The clinical parameters were controlled 3 months after surgery (PD, CAL, KT), and the results are shown in Table 2. Means  $\pm$  standard deviations of PD, CAL, and KT (middle point) at baseline, at 3 months after surgery, and the differences are reported in Table 3.

An increased amount of gingiva was obtained in all the treated sites. The newly formed keratinized tissue was well integrated into the adjacent gingiva, with excellent esthetic results. The histological features showed fully keratinized tissue in all the treated sites. Another case (case #1) is illustrated in Figure 7.

## DISCUSSION

Tissue engineering represents one of the most exciting advances in regenerative medicine. The ability to produce new tissue and organs from a patient's own cells has changed treatments and prognoses for many patients.

One of the most commonly used applications of tissue engineering is in dermatology. In these cases, covering exposed granulation tissues of large skin wounds using dermal-epidermal cells (taken directly from the patient) supported by resorbable membranes allows for significant improvements in the patient's prognosis. It is also important to note that the material is obtained from a small amount of undamaged skin in a relatively short time (7 to 8 days).

In periodontology a similar, but less dangerous, clinical situation is represented by the use of a similar procedure on denuded periosteum for gingival augmentation. In traditional gingival augmentation procedures, a large epithelial/connective tissue graft is used to cover the exposed periosteum to provide the necessary amount of keratinized tissue. Such gingival augmentation often results in an excessive amount of unsightly keratinized tissue on the treated area due to the irregularity of the superficial appearance of the tissue and the discontinuation/misalignment of the mucogingival junction. In addition, removing tissue from the palate can cause the patient considerable discomfort.

**Table 2.**  
**Three-Month Measurements of Treated Sites**

Patients	Gender	Age	Tooth	3-Month Postoperative				
				PD (mm)	CAL (mm)	KT (mm)		
						Mesial	Middle	Distal
1	F	38	#22	1.0	1.0	3.5	3.0	3.5
2	M	34	#27	1.5	1.5	3.0	2.5	2.5
3	F	24	#20	1.5	5.0	3.5	3.0	3.0
4	F	38	#27	1.0	1.5	3.5	2.5	3.5
5	M	32	#3	2.0	3.5	4.0	3.0	3.0
6	F	35	#24 #25	0.5 0.5	4.0 4.0	3.5 3.0	3.0 3.5	3.5 4.0

**Table 3.**  
**Means, Standard Deviations, and Differences Between Baseline and 3 Months After Surgery**

Parameters	Baseline	3 Months	Difference
PD	1.00 ± 0.50	1.14 ± 0.56	0.14 ± 0.24
CAL	2.86 ± 1.65	2.93 ± 1.57	0.07 ± 0.19
KT in middle site	0.93 ± 0.34	2.93 ± 0.34	2.00 ± 0.41

The aim of this study is to show how a technique based on tissue engineering principles can be applied to periodontology and to highlight its clinical advantages in cases where gingival augmentation is necessary. The process of taking a very small portion of gingiva (2 × 1 × 1 mm), cultivating gingival fibroblasts, and receiving the cultivated cells in a short period of time (15 days) has been successfully tested in a previous case report.<sup>10</sup>

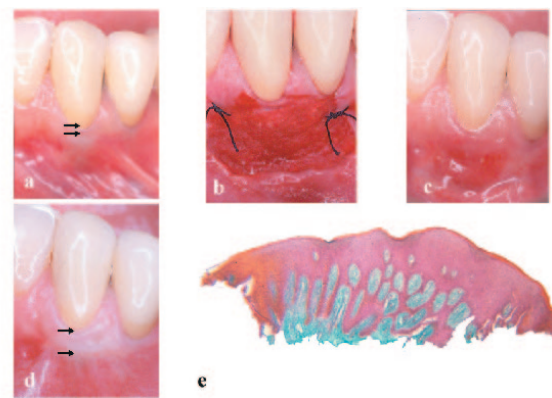
The results of the present study show an increased amount of keratinized tissue on all treated sites after 3 months (Tables 2 and 3). The mean average of the increased amount of keratinized tissue was 2.00 ± 0.41 mm (range 1.5 to 2.5 mm) in the middle site (which is the most important measurement point for the graft) of the treated teeth. This outcome could appear smaller in terms of apico-coronal augmentation of gingiva, if compared

to outcomes obtained by means of free gingival graft procedures. Nevertheless, the increased amount of gingiva obtained permitted satisfactory plaque control in the patients. In addition, excellent esthetic results were achieved in the treated areas because of the complete integration of the newly formed gingiva, along with a maintenance of the level of the mucogingival junction.

The clinical results were confirmed by histological evaluation performed after 3 months; all cases showed a keratinized epithelium supported by dense connective

tissue. The HA membranes were not histologically present.

Unlike the technique used in dermatology in which both epithelium and connective cells are utilized to cover the exposed “derma,” in our patients, only cultivated gingival fibroblasts without keratinocytes supported by resorbable HA scaffold were directly applied to the exposed periosteum. This is because it has



**Figure 7.**

Case #1. **a)** A new crown is scheduled for this lower left canine. Gingival augmentation is needed before the prosthetic rehabilitation because of the extremely reduced amount of gingiva (arrows). **b)** The HA membrane with the patient-cultivated fibroblasts is adapted and fixed onto the recipient site. **c)** Healing after 20 days. **d)** The apico-coronal dimension of the gingiva is increased (arrows) after 3 months. **e)** Histological examination shows full keratinization of the newly formed tissue after 3 months (Masson's stain; original magnification ×60).

been demonstrated that keratinization of the gingival epithelium is controlled by the morphogenetic stimuli of underlying connective tissue.<sup>11,12</sup> Therefore, the cultivated keratinocytes were not needed to guarantee subsequent keratinization.

Based on these clinical cases, the tissue engineering technique offers several advantages if applied in cases of gingival augmentation: 1) a very small donor site (2 × 1 × 1 mm); 2) adequate amount of keratinized tissue obtained; 3) fine esthetic results; and 4) minimal discomfort for the patient.

For the time being, the only disadvantage of this technique in periodontal therapy is that it is limited to mucogingival surgery, namely, gingival augmentation procedures. In fact, the cultivated gingival fibroblasts and keratinocytes that are cultured onto a non-woven HA matrix need an adequate blood supply for survival. During gingival augmentation, the exposed periosteum is an excellent vascular recipient for the grafted cells. This limitation currently reduces the utilization of tissue engineering techniques to the treatment of gingival recessions in which the exposed avascular root surfaces do not allow the cultivated cells to survive.

Regarding the risk of disease transmission (bovine spongiform encephalopathy) related to the use of fetal calf serum during the culturing period, the infection is avoided by using FCS obtained from certified North American animals that are further certified, according to European standards, by Fidia Advanced Biopolymers as "transmissible spongiform encephalopathy-free."

In conclusion, the tissue engineering technique may be considered a new gingival augmentation procedure. Of course, further controlled clinical testing is needed.

### ACKNOWLEDGMENTS

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Correspondence: Prof. Giovan Paolo Pini Prato, Viale Matteotti 11, 50121 Florence, Italy. Fax: 39 05 5572881; e-mail: gpinipr@tin.it.

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