

CASE REPORT

Tissue-Engineered Oral Mucosa for Mucosal Reconstruction in a Pediatric Patient With Hemifacial Microsomia and Ankyloglossia

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Many types of soft tissue grafts have been used for the reconstruction of oral mucosal defects. The best results are achieved with mucosal grafts; however, when large areas must be grafted, sufficient donor tissue is not available. Tissue engineering represents an alternative method to obtain sufficient autologous tissue for reconstructing oral wounds. Herein we present a pediatric patient with hemifacial microsomia and congenital ankyloglossia requiring multiple surgical interventions, and in which an autologous full-thickness tissue-engineered oral mucosa was used for successful oral reconstruction. Our study demonstrates that even under challenging conditions, robust tissue-engineered products, such as the fibrin-based oral mucosa described here, can achieve successful tissue regeneration.

KEY WORDS: *ankyloglossia, hemifacial microsomia, keratinocytes, scaffold, tissue engineering*

Hemifacial microsomia (HFM) is the most common facial congenital disability after cleft lip and palate and yet its etiology is not fully understood. The incidence of this rare disease has been reported to range from 1:3500 to

1:45,000 live births, depending on the study (Balaji, 2010; Gawrych et al., 2011). The clinical manifestations are multiple and varied, but the appearance of the patients is characterized by facial asymmetry and ear defects. This is due to the unilateral hypoplasia of the craniofacial skeleton and its overlying soft tissues (Gougoutas et al., 2007). The patient described in this paper had HFM and other oral soft tissue anomalies that required mucosal reconstructive procedures.

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Several autologous soft tissue grafts have been used for oral mucosal reconstruction, including full- and split-thickness grafts, and oral mucosa from the palate. A disadvantage of full-thickness skin grafts in the oral cavity is that they frequently exhibit hair growth and keratinization (Raguse and Gath, 2005). Split-thickness skin grafting is a simple surgical technique, but the postoperative functional condition of the intraoral lining is often poor (Lauer and Schimming, 2001). Better results are achieved with mucosal grafts, but adequate amounts of donor tissue may not be available when large areas must be grafted. All grafts have the disadvantages of donor site morbidity, postoperative pain, risk of infection, or surgical complications.

One alternative to autologous soft tissues transplantation is the use of commercial dermal replacements (Raguse and Gath, 2005). Good outcomes using these products have been reported in selected cases, but to our knowledge, there is scarce information about the use and efficacy of these substitutes for intraoral mucosal lining. The cost of these products can be high and may preclude their widespread use.

Tissue engineering opens the possibility of growing living tissues *in vitro* for grafting. Several attempts have been

made to transplant tissue-engineered oral mucosa in reconstructive procedures (Lauer and Schimming, 2001; Sauerbier et al., 2006). Although good clinical results have been achieved with this engineered tissue, some wound shrinkage was observed. This fact may be due to the absence of submucosal connective tissue since these cultured mucosal grafts consist only of a thin layer of keratinocytes (Lauer and Schimming, 2001).

In this study, we assessed the efficacy of a novel tissue-engineered oral mucosa in a pediatric patient with HFM and congenital ankyloglossia. The full-thickness engineered autologous oral mucosa consisted of both submucosal connective tissue and epithelium (Peña et al., 2010, 2011) and was successfully used to reconstruct a complicated, multi-operated oral mucosal defect. A stable long-term outcome was achieved.

CASE PRESENTATION

The patient initially presented as a 1-year-old patient who was referred to the Oral and Maxillofacial Surgery Department of the Hospital Universitario 12 de Octubre (Madrid). Physical examination at that time revealed multiple facial and oral anomalies including right ear lobe hypoplasia, right mandibular hypoplasia with multiple dental agenesis, right soft palate paralysis and velopharyngeal incompetence, hypoplastic right facial soft tissue, and mandibular branch of right facial nerve paralysis, leading to a diagnosis of HFM type 2a. Relevant to this report, the patient also had absence of the right buccal vestibule, gingival margin, and lateral floor of the mouth, resulting in ankyloglossia (Fig. 1).

The first surgical attempt to free the tongue was performed at the age of 4 years. A longitudinal incision along the right lateral margin of the tongue was performed, freeing the tongue from the alveolus. No substitute tissue or splint was used to line or cover the surgical wound. The mucosa over the mandibular ridge healed by secondary epithelialization. An effective freeing of the tongue was achieved, with good anterior projection and lateral mobility. Unfortunately, with healing and scar production, the ankyloglossia recurred within the first few weeks of follow-up.

One year after this first attempt, a second surgical freeing of the tongue was performed. This time an acellular dermal matrix graft (Alloderm, BioHorizons, Birmingham, AL.) was used to line the surgical wound, but without any protective splint covering the defect. This graft failed, and the ankyloglossia again recurred in the immediate postoperative period.

After these two successive failures and given the compromised status of the surgical defect, the option of using autologous cultured oral tissue was considered. Written informed consent was obtained from the patient's family, and the procedure was approved by the Coordinator of Transplants and Investigation Ethics Committee of



FIGURE 1 Initial appearance of the oral anomalies before surgical reconstruction. A band in the right buccal mucosa can be observed (arrow). Note also the palatal and mandibular asymmetry.

Hospital Universitario 12 de Octubre as required by the Helsinki Declaration principles.

Sources of Cells for Culture From Oral Mucosa Biopsy

In order to obtain keratinocytes and fibroblasts to tissue-engineer the full-thickness oral mucosa (Peña et al., 2010, 2011), a 5 mm² tissue specimen was harvested from the patient's buccal mucosa. The specimen was sent to the Tissue Engineering Unit of our center (Centro Comunitario de Sangre y Tejidos del Principado de Asturias). The tissue was minced and enzymatically digested with collagenase solution (2 mg/mL) (Sigma, St. Louis, MO.) for 1.5 to 3 hours. After collagenase digestion, the solution was centrifuged at 400 × g for 10 minutes. The pellet was resuspended in fibroblast culture medium (DMEM, Gibco, Paisley, U.K., supplemented with 10% FCS, Gibco, Auckland, NZ). The remaining skin fragments were digested secondly with trypsin (0.05%)/EDTA (0.02%) (Lonza, Verviers, Belgium) (T/E) for 30 to 60 minutes. The removed T/E was centrifuged at 400 × g for 10 minutes. Two more trypsin digestions were performed for 30 minutes each on the undigested tissue pellet. The pellets were then resuspended in keratinocyte culture medium.

Fibroblasts Culture

Cells obtained from collagenase digestion were seeded ($1 \times 10^5/\text{cm}^2$) using fibroblast culture medium (primary fibroblast culture) and maintained at 37°C in a 5% CO_2 incubator. When an increase in the fibroblast-like cells was observed, secondary cultures were prepared. Cells were seeded to yield a twofold increase in surface. The secondary culture was maintained until keratinocyte subconfluence was achieved. Fibroblasts were then detached from the flask by T/E treatment and used to generate the plasma-based and fibroblast scaffold and frozen for later use.

Keratinocytes Culture

Cells obtained from trypsin digestions, as described above, were seeded (2.5×10^4 cells/ cm^2) in the presence of $1 \times 10^5/\text{cm}^2$ lethally irradiated 3T3 (European collection of animal cell culture, no. 85022108) and cultured following the method initially described by Rheinwald and Green (1975). The keratinocyte culture medium was a mixture of DMEM (Gibco)/HAM-F-12 (Gibco) supplemented with 10% FCS (Gibco), EGF (Austral Biologicals, San Ramon, CA), insulin (Sigma), cholera toxin (Sigma), hydrocortisone (Sigma), triiodothyronine (Sigma), and adenine (Sigma) as previously described (Llames et al., 2004). Subconfluent primary keratinocyte cultures were treated with T/E and propagated on the engineered plasma-based scaffold and frozen for later use.

Preparation of Plasma-based and Fibroblast Scaffold

A plasma-based scaffold filled with fibroblasts was used as a submucosal component of the bioengineered oral mucosa, in a way similar to that previously reported by our group for skin (Llames et al., 2004, 2006) (PCT/ES 2002/00087, WO 2002/072800). Fresh frozen plasma was obtained from voluntary donors of the local blood bank (C.C.S.T. Asturias). Plasma was measured for fibrinogen concentration with a hemostasis testing system (ACL Top 700, Instrumentation Laboratory, Bedford, MA). Between 45 and 55 mg of fibrinogen was used to synthesize the plasma-based scaffold. Then, 5×10^4 cultured fibroblasts were resuspended in the appropriate quantity of plasma containing 20 mg of tranexamic acid (Amchafibrin, Rotthafarm Madaus, Barcelona, Spain) and finally, 4 mL of CaCl_2 (Sigma) 1% was added to initiate the coagulation of the mixture. The mixture was placed in a tissue culture flask (75 cm^2) and allowed to solidify at 37°C in a 5% CO_2 incubator for 30 minutes. Once the plasma-based fibroblast-containing scaffold clotted, it was covered with culture medium. When primary culture of keratinocytes reached confluence, cells were

detached and seeded over the plasma-based scaffold surface at a density of 25×10^3 cells per cm^2 .

Preparation of the Full-thickness Tissue-engineered Oral Mucosa for Grafting

Once the mucosal keratinocytes were confluent over the plasma-based scaffold, the full-thickness tissue-engineered mucosa was prepared for grafting as previously described (Meana et al., 1997; Peña et al., 2010). The culture flask was opened, the culture medium was removed, and the bioengineered mucosa was fixed to a nonpetroleum gauze with Histoacryl (B. Braum, Melsungen, Germany) (Meana et al., 1997). The tissue-engineered mucosa was manually detached from the culture flask using a plastic spatula, rolled up and introduced in a 50-mL tube with DMEM, and sent to the Hospital Universitario 12 de Octubre by an express courier.

RESULTS

The mucosal keratinocytes reached confluence over the plasma-based scaffold 14 days after seeding, and the full-thickness tissue-engineered oral mucosa was grafted. The tongue was freed from the mandibular ridge by making an incision along the right lateral border of the tongue. The intrinsic musculature of the tongue inserting into the lingual surface of the mandible was dissected free along with the fibrosis produced by the previous surgeries. Once the tongue was freed, it was able to be mobilized anteriorly beyond the vermilion of the lower lip. After careful hemostasis, the full-thickness tissue-engineered oral mucosa was adapted and sutured to the lingual mucosal defect. A tooth-supported acrylic splint was made preoperatively that mirrored the contralateral arch of the mandible in order to protect the graft and to help secure its position in the glossomandibular sulcus (Fig. 2). The prosthesis was kept in place for 4 weeks with the hope of avoiding retraction of the graft and the early recurrence of ankyloglossia.

After this first grafting of engineered mucosa, the surgical results were satisfactory, with an effective freeing of the tongue. This state was maintained for the first few months of follow-up; however, subsequent progressive contracture of the wound during the healing period resulted in the partial recurrence of ankyloglossia (Fig. 3). By the end of the first postoperative year, it was deemed necessary to perform a second graft. A new full-thickness oral mucosa was tissue-engineered using the same protocol and the patient's cryopreserved cells. The use of cryopreserved cells did not alter any cell growth or physical characteristic of the product (data not shown). The secondary autologous mucosal equivalent was grafted following the same procedure described above. This procedure was finally successful, and resulted in good long-term tongue mobility



FIGURE 2 Full-thickness tissue-engineered oral mucosa prepared for grafting. The tissue-engineered mucosa is attached to a nonpetroleum gauze to facilitate its manipulation. The acrylic splint, used to fix the graft to the glossomandibular sulcus, is also shown (arrows).

that has been maintained for the 2-year follow-up period of the study. Although tongue function is normal, a small area of residual fibrosis has persisted posteriorly, but has resulted in no functional impairment of the tongue (Fig. 4).

The patient also eventually underwent Furlow palatoplasty and pharyngeal flap surgery for the treatment of velopharyngeal insufficiency. This, together with the tongue surgery has given the patient a good overall result. Evaluation by speech therapists has shown satisfactory speech, and the patient has not had any academic delays



FIGURE 3 Partial recurrence of the ankyloglossia. One year after the first grafting procedure a partial recurrence of the ankyloglossia was observed along the right edge of the mandible. A band is shown (arrow).



FIGURE 4 Final appearance of the reconstructed tissue 2 years after the second full-thickness tissue-engineered oral mucosa grafting procedure. A minimal residual area of fibrosis persists along the right lateral posterior border of the tongue (arrow), without limiting its anterosuperior projection.

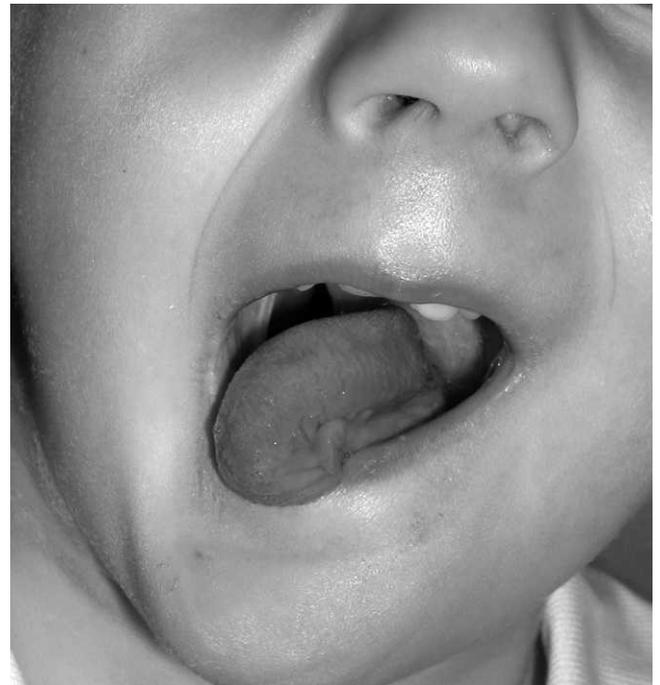


FIGURE 5 Current appearance of the tongue. The effective release of the tongue can be noticed. Lingual lateral deviation is the result of a congenital paresis of the hypoglossal nerve, which can be observed in some cases of HFM.

secondary to the defects themselves or corrective surgeries (Fig. 5).

DISCUSSION

This case report details our experience treating a pediatric patient with ankyloglossia and HFM through the use of tissue-engineered oral mucosa. Because of the age of the patient and problems with multiple episodes of recurrence of the condition, the therapeutic approach was challenging.

The first attempts of reconstruction made with no substitute or with acellular dermal matrix, did not solve the problem and the ankyloglossia recurred. There are several possible reasons why these surgeries may have failed, including hematoma, poor fixation of the Alloderm graft, lack of a splint, or a combination of these potential problems. It is our opinion, however, that the likely explanation for these failures is the absence of epithelial tissue responsible for the definitive closure of the wound.

Better esthetic and functional outcomes can be achieved when autologous oral mucosa is used for the reconstruction of oral defects (Sauerbier et al., 2006). There are, however, several potential shortcomings associated with the use of autologous mucosa, including donor site morbidity and insufficient tissue for large wound coverage (Lauer and Schimming, 2001). This can be especially problematic if the patient is an infant or small child.

Several authors have reported the use of cultured mucosal keratinocyte sheets for oral reconstruction (Lauer and Schimming, 2001; Sauerbier et al., 2006). These epithelial sheets are reported fragile and difficult to manipulate. Once they are grafted, poor epithelial organization and some wound shrinkage are observed. This might be because these cultured mucosal grafts consist only of a thin epithelial layer without any underlying submucosal connective tissue. The connective tissue component seems to improve handling, intraoral placement, and stabilization of the graft at the surgical site (Peramo et al., 2012). The connective tissue portion of these engineered constructs is a three-dimensional scaffold populated by fibroblasts that resembles a natural lamina propria and lends substance to the graft and facilitates manipulation. Aside from making the graft easier to use, the subepithelial fibroblasts play an important role in epithelial morphogenesis, keratinocyte adhesion, and the formation of the complex dermoepidermal junction needed for tissue maturation and stability (Peña et al., 2010).

Many natural biomaterial-derived scaffolds have been used in oral mucosal reconstruction including collagen, amniotic membrane, gelatin-based materials, and fibrin (Moharamzadeh et al., 2007; Peña et al., 2010, 2011, 2012). Natural materials possess several characteristics that make them popular as scaffolds for tissue engineering. However, these naturally derived materials also have some disadvantages. Many of them are not available in large quantities, they may suffer from large batch-to-batch

variations, and they are typically expensive (Moharamzadeh et al., 2007).

Our group has been working with fibrin-based and fibroblast dermal scaffolds since 1998 (Meana et al., 1998) and have used them successfully for the treatment of severe skin conditions including extensive burns (Llames et al., 2004; Gómez et al., 2011), the resection of giant nevi, graft versus host disease, and defects resulting from the resection of neurofibromas (Llames et al., 2006). This fibrin-based and fibroblast scaffold has also been used for oral reconstruction in different conditions such as epidermoid carcinoma, pleomorphic adenoma, and epulis fissuratum (Peña et al., 2012).

Fibrin is an ideal matrix because it can stimulate fibroblast proliferation while preserving the epidermal cell “stemness” (Del Rio et al., 2002). Once it is transplanted, fibrin matrix is replaced by collagen fibers synthesized *de novo* by the embedded fibroblast population and forms tissue histologically similar to a natural lamina propria (Peña et al., 2011).

The occurrence of wound contracture observed after the first transplant of full-thickness engineered mucosa led us to perform a second procedure 1 year later. This shrinkage may have been due to the contractile function of the fibroblast in the wound, or alternatively, it is possible that the number of epithelial stem cells contained in the tissue-engineered graft was not sufficient to achieve the persistent regeneration of the tissue. These hypotheses need to be investigated further.

The second graft was prepared with cryopreserved cells. This is another advantage of the technique since no additional tissue harvest was needed, and further morbidity for the patient was avoided. With this second graft, the ankyloglossia was resolved, and normal function and mobility of the tongue was established.

The full-thickness tissue-engineered mucosa described here opens the possibility of synthesizing a completely autologous full-thickness oral mucosa (Peña et al., 2012), using autologous plasma for tissue engineering the plasma-based component of the connective tissue scaffold. In this case, the age of the patient did not allow for the collection of a sufficient quantity of autologous blood necessary to synthesize the scaffold. However, as it has been done with bioengineered skin (Llames et al., 2004), when the size of the patient is sufficient to allow the collection of enough plasma, the creation of a completely autologous full-thickness tissue-engineered oral mucosa (scaffold and cells) is possible. As an additional benefit, if further transplantation is required, cells previously harvested and cryopreserved can be used to create new tissue for grafting.

This model of full-thickness tissue-engineered mucosa is inexpensive, donor cells are easy to obtain, and the tissue created can be ready for use in 3 weeks. Considering that most of the indications for the use of oral mucosal grafts do not involve immediately life-threatening conditions, this time period seems reasonable.

Overall, our studies demonstrate the feasibility of using the tissue-engineering approach for this and other challenging conditions. Further research and refinement of techniques should help define the place of this promising technology in the armamentarium of the reconstructive surgeon.

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