

Implantation of tissue-engineered mucosal substitutes in the dog palate

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SUMMARY Tissue shortage complicates the surgery of cleft palate (CP) anomalies. The healing of defects on the palate impairs growth of the dento-maxillary complex due to scar tissue formation. Implantation of grafts into the wound area might reduce this adverse effect of surgery. The aim of this study was to evaluate a cultured autologous mucosal substitute, which can be used as a graft material. Two different types of cultured mucosal substitutes composed of skin-derived substrates (unprocessed dermis and AlloDerm®) and autologous oral keratinocytes were implanted in palatal wounds in six beagle dogs (1–1.5 years of age). The cultured substitutes were compared with a sham and a control group. The animals were sacrificed in pairs 1, 3, and 12 weeks after surgery. Epithelial regeneration, inflammatory response (leucocyte protein L1), ingrowth of (myo-) fibroblasts, collagen type III, and formation of a basal membrane (JM 403) were evaluated.

The results demonstrated that all cultured substitutes possessed a multilayered epithelium, closely resembling normal palatal epithelium. After implantation, however, the epithelium was lost and an inflammatory response was observed in the first week. After 3 and 12 weeks, the implanted substitutes had completely disappeared and epithelial migration occurred from the wound margins.

It is possible to culture an autologous epithelium on a skin-derived substrate and implant it as an oral mucosal substitute in palatal wounds. However, these substitutes do not improve the healing of palatal wounds. It is suggested that the revascularization of the wound area is too slow to allow survival and integration of the substitutes.

Introduction

Oral mucosa defects in the lateral areas of the palate are often an inevitable consequence of cleft palate (CP) surgery. These wounds heal by secondary intention involving wound contraction and scarring. Extensive scar tissue is formed that is firmly anchored to the palatal bone. Although CP surgery has advanced, scar tissue is still a major factor in the impairment of maxillary growth and the development of the dentition in CP patients (Ross, 1987; Berkowitz, 1996; Ishikawa *et al.*, 1998).

Animal studies have shown that manipulation of the mucoperiosteum leads to dento-alveolar growth inhibition. The collagen fibres of the scar tissue are orientated in a transverse direction and attached to the underlying bone by Sharpey's fibres. It was suggested that prevention of scar tissue attachment to the palatal bone might lead to more favourable maxillary growth (Searls *et al.*, 1979; Wijdeveld *et al.*, 1991). In beagle dogs, wound contraction occurred during the first weeks after wounding, and was associated with the appearance of myofibroblasts that are involved in wound contraction (Searls *et al.*, 1979; Squier and Kremenak, 1980; Desmouliere, 1995). These cells contribute to collagen accumulation and therefore to scar tissue formation. In the later phases of wound healing, the myofibroblasts disappear, presumably through apoptosis,

and wound contraction ceases (Desmouliere, 1995). The wound tissue is then slowly remodelled into a mature scar. Therefore, reducing the number of myofibroblasts during wound healing and prevention of the tissue attachment to the palatal bone after cleft closure might decrease impairment of maxillary growth and development of the dentition.

Studies in a full-thickness skin wound in animal models on the use of grafts demonstrated a reduction in the number of myofibroblasts (Rudolph, 1979), which may result in decreased wound contraction and scar tissue formation. For the reconstruction of small defects in the oral cavity, the use of palatal or buccal mucosal autografts has been described (Hertel *et al.*, 1993; Simons *et al.*, 1993), but for larger oral defects, it is impossible to obtain sufficient mucosa. Autologous skin grafts have also been employed in animal and human studies, but they seem to maintain their original characteristics such as hair growth (Sanders and McKelvy, 1976; Endo *et al.*, 2001). Furthermore, autologous grafts are associated with donor-site morbidity.

In vitro culture techniques have been used to increase the availability of oral keratinocytes (Lauer, 1994). However, grafts composed of only cultured keratinocytes are fragile, difficult to handle, and when grafted on a full-thickness intra-oral wound, undesirable wound contraction still occurs (Lauer and Schimming, 2001).

Two types of dermal substrates, collagen-based and skin-derived materials, have been used for intra-oral grafting. The collagen-based substrates generally consist of a type I collagen matrix, sometimes supplemented with other extracellular matrix (ECM) components such as elastin or glycosaminoglycans (Ellis and Yannas, 1996; Fujioka and Fujii, 1997; van Zuijlen *et al.*, 2000). The skin-derived substrates consist of glycerol-preserved cadaveric skin, or skin that is processed to remove cellular components (Rennekampff *et al.*, 1997). The latter technique leads to a relatively immunologically inert allograft. Skin-derived substrates, such as AlloDerm®, have been used for intra-oral applications (Batista and Batista, 2001; Clark *et al.*, 2003). These dermal substrates diminish the impairment of maxillary growth if applied to the denuded bone surfaces following CP repair.

Full-thickness grafts composed of cultured keratinocytes and a dermal component grafted in skin defects showed faster re-epithelialization, less wound contraction, and less scar tissue (Clugston *et al.*, 1991; Kangesu *et al.*, 1993; Gustafson and Kratz, 1999). Both the cultured epithelium and the dermal substrate seem to contribute to these effects (Walden *et al.*, 2000). However, the intra-oral implantation of a full-thickness substitute in an open wound has not yet been investigated. Therefore, the overall aim of the present study was to develop a full-thickness substitute composed of a dermal substrate and oral keratinocytes for CP repair in order to minimize contraction and subsequent scarring.

Recently, it has been shown that substitutes composed of oral keratinocytes cultured on skin-derived dermal substrates possess histological and immunohistochemical characteristics close to normal oral mucosa (Ophof *et al.*, 2002). In the present study, two types of skin-derived substitutes were implanted in full-thickness wounds in the palatal mucosa of dogs. Histology and immunohistochemistry were performed to evaluate the tissue response and the wound healing process at 1 (inflammatory phase), 3 (granulation tissue and contraction phase), and 12 (remodelling phase) weeks after surgery. Inflammatory response, ingrowth of fibroblasts, re-epithelialization, and the formation of a basal membrane were evaluated.

Material and methods

Animals

Six beagle dogs (1–1.5 years of age) were used. All animals were kept under normal laboratory conditions and were fed standard dog chow and water *ad libitum*. The experiment was approved by the Board for Animal Experiments of the University of Nijmegen, The Netherlands.

Primary keratinocyte cultures

Dog oral keratinocytes were cultured as described previously (Ophof *et al.*, 2002). In short, 3 mm biopsies from the

palatal mucosa were obtained from each beagle dog. The biopsies were incubated in 0.25 per cent trypsin solution (Gibco, Grand Island, New York, USA) to detach the epithelium. A single-cell suspension was seeded onto a pre-plated feeder layer of lethally irradiated 3T3 fibroblasts in keratinocyte medium. When the cultures were nearly confluent, the keratinocytes were harvested and stored in liquid nitrogen in keratinocyte medium with 10 per cent dimethyl sulphoxide until use. Before every experiment, the cryopreserved autologous cells were quickly thawed at 37°C under gentle agitation. For culture of the substitutes, third-passage cells were used. Mycoplasma contamination is one of the main problems in cell cultures in biological and medical research, because mycoplasma can alter nearly all parameters and products of the cell; therefore, all cultures were checked for mycoplasma contamination using a mycoplasma kit (Gen Probe, Biomérieux Inc., St Louis, Missouri, USA).

Dermal substrates

Two different skin-derived dermal substrates were used, unprocessed de-epidermized dog dermis (DED) and commercially available processed human donor dermis (AlloDerm®, LifeCell Corporation, Branchburg, New York, USA; Batista and Batista, 2001).

DED was produced from full-thickness skin explants, as described previously for human skin (Ghosh *et al.*, 1997). The explants were obtained from the groin of beagle dogs, which is the least hairy region of the animal. Briefly, the skin was subjected to at least three freeze–thaw cycles using liquid nitrogen. Subsequently, the skin was placed in phosphate-buffered saline (PBS) supplemented with 100 U/ml penicillin G and 100 µg/ml streptomycin (Life Technologies, Breda, The Netherlands) at 37°C for 2 weeks to detach the epithelium. The DED was stored in PBS with antibiotics at –20°C until use. Both skin-derived substrates were washed three times in PBS before use.

Composite cultures

The skin-derived substrates (DED and AlloDerm®) were placed on stainless steel grids in keratinocyte medium. Subsequently, approximately 2×10^5 third-passage autologous keratinocytes were seeded on these substrates within a stainless steel ring, with an inner diameter of 10 mm that was placed on the substrate. The cells were cultured submerged in keratinocyte medium for 3 days and then air-exposed for a further 11 days. Before implantation, a small punch sample from the cultures was taken for histological evaluation.

Implantation

The six dogs were randomly assigned to three groups, for evaluation, at 1, 3, and 12 weeks after surgery. In each dog,

four standardized full-thickness wounds were created in the medial region of the hard palate down to the palatal bone with a 6 mm biopsy punch (Stiefel Laboratorium, Offenbach am Main, Germany).

Prior to surgery, the animals were premedicated with 0.5 ml Thalamonal® (0.05 mg Fentanyl and 2.5 mg/ml droperidol; Janssen Pharmaceutica, Beerse, Belgium) and 0.5 ml atropine intramuscularly (0.5 mg/ml atropine sulphate). Subsequently, they were anaesthetized with an intravenous injection of 30 mg/kg Narcovet® (60 mg/ml sodium pentobarbital; Apharmo, Arnhem, The Netherlands). After intubation, anaesthesia was maintained with Ethrane® (15 mg/ml enflurane; Abbott, Amstelveen, The Netherlands). The palatal mucosa was cleaned with Betadine® solution (povidoniodine; Dagra-Pharma, Diemen, The Netherlands). In addition, approximately 1.5 ml Xylocaine® (0.4 mg/ml lidocaine-hydrochloride and 0.0125 mg/ml adrenaline; Astra Chemicals, Rijswijk, The Netherlands) was injected into the palatal mucosa to avoid excessive bleeding during the procedure.

The circular, non-critical size, soft tissue defects were made distally to the canines, and then randomly assigned to one of the following treatments: (1) left open (control); (2) filled with an autologous mucosal punch biopsy from one of the other wounds (sham); (3) filled with DED-based culture; and (4) filled with Alloderm-based culture. Every implant was fixed gently in place using four stitches (4-0 Vicryl, Ethicon; Johnson & Johnson Company, Amersfoort, The Netherlands). A pressure dressing was not used because these materials have been shown to induce persisting inflammation and cytotoxic effects, which may result in a delay in wound healing (Smeekens *et al.*, 1992; Alpar *et al.*, 1999).

After surgery, the animals were medicated with 1.5 ml of Albipen® 15 per cent (ampicillin anhydrate 150 mg/ml; Mycofarm, de Bilt, The Netherlands). All animals received a normal diet after surgery.

Histology

At the time of sacrifice, the animals were brought under general anaesthesia using 30 mg/kg Narcovet®. After some minutes, a lethal dose of Narcovet® was injected intravenously. A small mucosal biopsy (Ø 4 mm) was taken from the margin of each wound. These biopsies, containing tissue from the experimental wound and some normal palatal mucosa, were fixed for 4 hours in 4 per cent paraformaldehyde in 0.1 M PBS at room temperature and embedded in paraffin. The samples were used for immunohistochemical staining (see below).

Subsequently, the maxillae were dissected and immersed in 4 per cent paraformaldehyde in 0.1 M PBS at room temperature. After fixation, all biopsy wounds were dissected separately. Following decalcification, in 20 per cent formic acid and 5 per cent sodium citrate, the tissues were

dehydrated through a graded series of ethanol and embedded in paraffin. Serial sections, 7 µm, were made through the entire wound. Each tenth section was mounted onto a superfrost (Menzel-Gläser, Braunschweig, Germany) slide and stained with haematoxylin and eosin (HE) for a general tissue survey. For further histological analyses, selected sections from the centre of each wound were taken. Sirius red staining was used to visualize the collagen fibres, and elastin was detected with Weigert–Van Gieson staining the presence of the substrate (Lillie and Fullmer, 1976; Junqueira *et al.*, 1979). The sections were blindly evaluated by one author (RO) for the degree of inflammation (HE), epithelial regeneration (HE), and orientation and amount of elastic fibres (Weigert–Van Gieson staining). The variables were scored on a scale from 0 (none) to 4 (abundant). The re-epithelialization was given a score from 0 to 4 [0, still open; 1, closed with a few cell layers (less than 10); 2, closed with many cell layers and no rete pegs; 3, closed with many cell layers and some rete pegs; and 4, closed with many cell layers and normal rete pegs. For each staining, 12 sections from the centre of each wound were evaluated, and the median score of these sections for each characteristic was used.

Immunohistochemistry

Paraffin sections of the 4 mm mucosal biopsies were collected on Superfrost Plus slides (Menzel-Gläser), the paraffin was removed and the sections were rehydrated again. Before staining, the slides were treated with 0.1 per cent trypsin 250 (DIFCO Laboratories, Detroit, USA) for 10 minutes and rinsed with PBS. Thereafter, the slides were treated with 3 per cent H₂O₂ in PBS for 30 minutes to block endogenous peroxidase and rinsed in PBS. All sections were pre-incubated with 5 per cent bovine serum albumin (PBSA; Sigma Chemical Co., St Louis, Missouri, USA) in PBS buffer.

Heparan sulphate. After pre-treatment, the sections were incubated with mouse anti-heparan sulphate IgM overnight at 4°C. After washing with PBS, the sections were incubated with a biotinylated goat anti-mouse antibody 1:400 (Vector Laboratories, Burlingame, California, USA) for 60 minutes at room temperature. After treatment with first and second antibodies, all sections were washed with PBS and treated with ABC peroxidase (Vector Laboratories). Peroxidase activity was visualized with a standard DAB technique (Sigma Chemical Co.). The sections were counterstained with Dellafields haematoxylin.

Collagen type III was detected with a rabbit anti-collagen type III antibody (Chemicon, Temecula, California, USA), and biotinylated goat anti-rabbit antibody was used as a secondary antibody (Chemicon). α -Smooth muscle actin was detected by a mouse anti-smooth muscle actin antibody (1:1600; Sigma Chemical Co.). The staining method was the same as described for heparan sulphate, and the

secondary antibody was biotinylated donkey anti-mouse antibody (1:500; Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA). Leucocyte protein L1 was detected with a mouse anti-L1 macrophage antibody (1:800; Abcam Limited, Cambridge, UK) with biotinylated donkey anti-mouse antibody as secondary antibody (1:500; Jackson ImmunoResearch Laboratories).

The general results are specified in Table 1. Representative sections were photographed on a Leitz DMRD Microscope (Leica, Wetzlar, Germany).

Results

Histology

Normal palatal mucosa. The palatal mucoperiosteum of a beagle dog consists of four layers: epithelium, lamina propria, submucosa, and periosteum; the first three layers are shown in Figure 1A. The palatal epithelium contains a basal layer with cuboidal and columnar cells. Superficial to the basal layer, about 15 layers of spherical to flattened cells are present with normal epithelial stratification. The parakeratinized layer is composed of flat cells in which often pyknotic nuclei are visible. The lamina propria contains a relatively loose network of collagen fibres while

in the deeper mucosa more densely packed collagen fibres are found. The submucosa contains the major arteries, veins, and nerves of the palate. Elastin is found in the submucosa and in the walls of the blood vessels of normal palatal mucosa. The mucoperiosteum is attached to the palatal bone by a thin periosteal layer containing some osteoblasts. Only a few collagen fibres connect the fibrous layer of the periosteum to the palatal bone.

Cultured mucosal substitutes in vitro. Oral keratinocytes cultured on both DED and AlloDerm® formed a parakeratinized epithelium, which was approximately 10 cell layers thick (Figure 1B). The epithelium contained a basal cell layer with cuboidal cells, some intermediate layers with vacuoles within the flattened cells, and a thick cornified layer of the parakeratinized type. The epithelium invaded the voids left by hair follicles. All these cultures resembled normal palatal epithelium of the beagle dog.

Experimental wounds

One week after implantation. A fibrin clot and many polymorphonuclear leucocytes were present in the untreated wounds (Figure 2A). The epithelium had started to proliferate and keratinocytes had migrated from the wound edges under part of the clot. The wounds were

Table 1 Semi-quantitative analyses of the experimental wounds.

	Control	Sham	DED	AlloDerm®	Control	Sham	DED	AlloDerm®
Weeks post-surgery	Degree of inflammation				Collagen type III			
1	1	2	2	2	1	1	1	1
3	1	1	2	1	1	1	2	1
12	0	0	0	0	3	2	2	2
	α -Smooth muscle actin				Leucocyte protein L1			
1	4	3	4	4	3	4	4	4
3	2	3	2	3	2	1	2	1
12	1	1	2	1	0	0	0	0

For each group, the median is shown after 1, 3, and 12 weeks post-surgery. The scoring system used for degree of inflammation, presence of collagen type III, α -smooth muscle actin, and leucocyte protein L1 was on a scale from 0 (none) to 4 (abundant). DED, de-epidermized dog dermis.

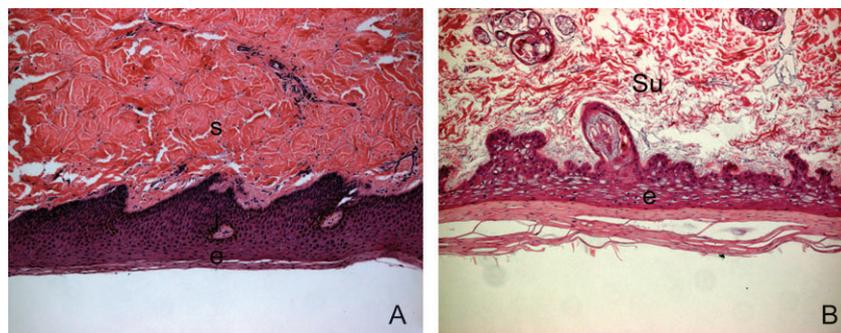


Figure 1 Histology of normal palatal mucosa and the cultured substitute. (A) Normal palatal mucosa of a beagle dog [haematoxylin and eosin (H&E)]. (B) Substitute containing oral keratinocytes on top of de-epidermized dog dermis cultured for 14 days (H&E). Su, substrate; e, epithelium; s, submucosa. Bar=50 µm.

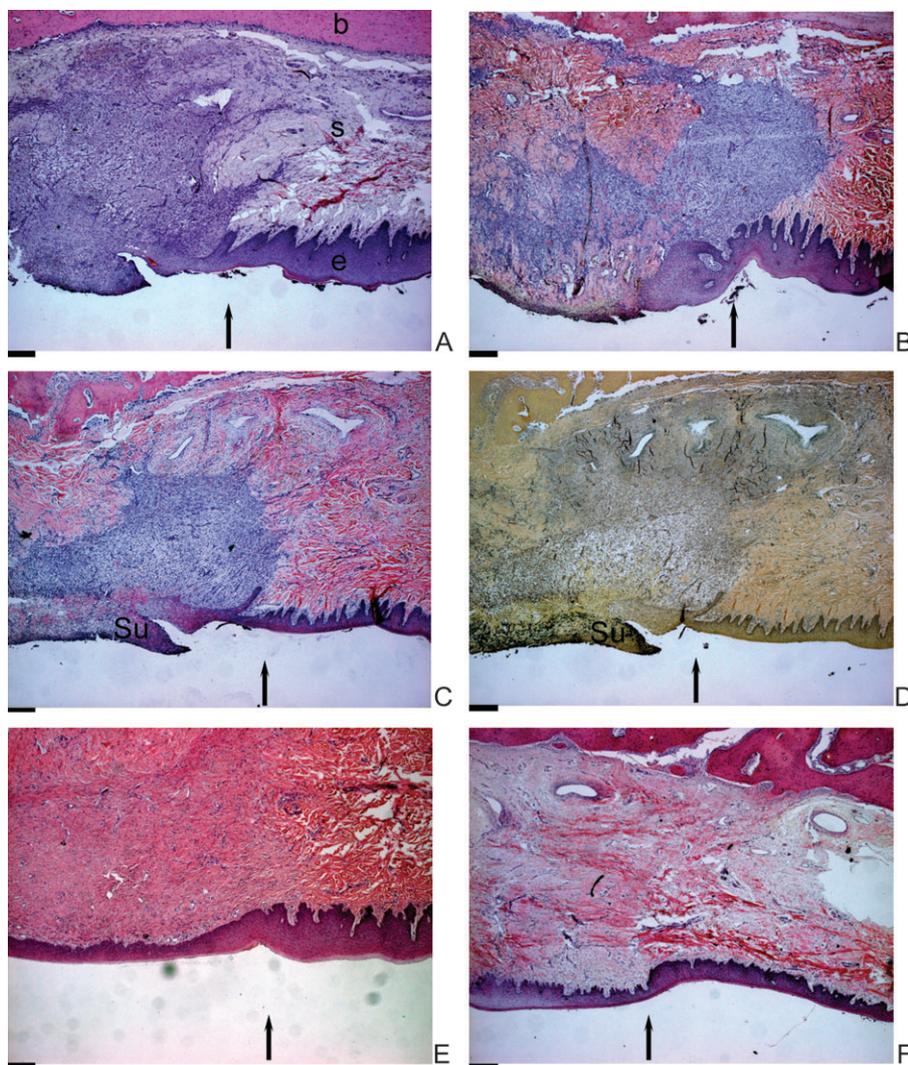


Figure 2 Histology of experimental wounds post-surgery. (A) Untreated wound 1 week after surgery [haematoxylin and eosin (H&E)]. (B) Autologous implant 1 week after surgery (H&E). (C) Cultured oral substitute (AlloDerm®) 1 week after surgery (H&E). (D) Cultured oral substitute (AlloDerm®) 1 week after surgery (elastin staining and elastin is stained black). (E) Cultured oral substitute (de-epidermized dog dermis) 3 weeks after surgery (H&E). (F) Cultured oral substitute (AlloDerm®) 12 weeks after surgery (H&E). Su, substrate; e, epithelium; s, submucosa; b, bone; and the arrow (↑) indicates the wound margin, the wound is on the left of this arrow. Bar=50 µm.

partly filled with granulation tissue. This tissue was rich in fibroblasts, polymorphs, and an extra cellular matrix (ECM) without a clear organization. Neither elastin nor collagen fibres were observed in the wounds. Superficial osteoclastic bone resorption was found on the palatal bone. The semi-quantitative analyses are summarized in Table 1.

The autologous biopsies could still be recognized in the pertinent wounds (Figure 2B). The dermal part of these biopsies was severely degraded, although elastin and collagen were clearly present, as patches in the outer part of the implant (not shown). The collagen fibres of the grafts were thick and resembled those of normal mucoperiosteum.

On top of the implant, tissue, rich in fibroblasts and granulocytes, was present, but no keratinocytes were found. The epithelium of the wound margins had started to proliferate and to migrate between the implant and the clot. Beneath the implant, tissue organization was similar to that of untreated wounds at 1 week after surgery.

The wounds filled with both types of mucosal substitutes showed similar histological characteristics as those filled with an autologous biopsy (Figure 2C). The cultured keratinocytes on top of the substrates were lost from all samples. The dermal part of the substitutes showed severe degradation. However, remnants of the substrate were always present in the superficial layer of the granulation

tissue as shown by the presence of elastin (Figure 2D). In addition, thick, mature collagen fibres of the substrate were still detected with Sirius red staining (not shown). Below this superficial layer, tissue was present with many fibroblasts, granulocytes, and an ECM with no clear organization. Neither elastin nor collagen fibres were found in this tissue. As in the untreated wounds, the epithelium had started to proliferate and had migrated from the wound edges under the superficial layer with the remnants of the implant.

Three weeks after implantation. The untreated wounds had closed with an epithelium composed of up to nine cell layers with a parakeratinized superficial layer (not shown). Rete pegs had not yet developed. The thin collagen fibres in the newly formed lamina propria had a mainly transverse orientation, and between these fibres, some inflammatory cells and many regenerating blood vessels were seen. Bone apposition was found at sites where the bone had probably been damaged by the biopsy punch (not shown). The semi-quantitative analyses are summarized in Table 1.

The tissue organization of the wounds filled with an autologous biopsy was comparable with that of the untreated wounds at 3 weeks. Furthermore, no elastin was found within the wound area. The wounds with the mucosal substitutes (Figure 2E) also showed the same histological characteristics as the untreated wounds. The substitutes could not be identified in any of the samples, as confirmed by a negative elastin staining. Between the thin parallel-orientated collagen fibres, some inflammatory cells were still present.

Twelve weeks after implantation. The histological findings for all wounds were similar (Figure 2F and Table 1). The epithelium had become thicker, although it had not regained its original thickness, and rete pegs were present. The connective tissue showed thinner collagen fibres than the tissue outside the wound area. The collagen fibres in the wound area were clearly aligned and elastin was absent. Inflammatory cells were no longer found in any of the samples. The periosteal layer in the wound area seemed to be thicker than outside this area. Collagen fibres penetrated into the newly deposited bone at the palate as Sharpey's fibres, thus creating an attachment of the wound tissue to the lamellar palatal bone (not shown).

Immunohistochemistry

Cultured mucosal substitutes in vitro. Collagen type III was observed throughout the dermal part of the cultures and a continuous staining for heparan sulphate was present at the epithelial–dermal border. α -Smooth muscle actin was not detected in the cultures. Leucocyte protein L1 was not expressed in the dermis, but the outer layers of the epithelium were always positive (not shown).

Experimental wounds

Heparan sulphate. Heparan sulphate staining was used to evaluate the presence of a basal membrane and angiogenesis in the wounds. Continuous staining for heparan sulphate was always observed at the epithelial–dermal border of the normal mucosa. Within the wounds, heparan sulphate staining of the basal membrane was not observed until 12 weeks after surgery (Figure 3A). Heparan sulphate positive regenerating blood vessels were observed in all wounds from 3 weeks after surgery.

Collagen type III. In normal palatal mucosa, an intensely stained network of loose collagen type III fibres was present in the lamina propria and around blood sinuses. Staining in the other regions of the submucosa was very weak. At 1 week post-surgery, all experimental wounds showed more intense collagen type III staining close to the surface than deeper in the wounds (Figure 3B). At 3 weeks after surgery, the intensity of the staining in the wound area had decreased and there were no differences between the experimental groups (Table 1). At 12 weeks post-surgery, the staining in the lamina propria and the periosteum in the wound area was close to normal, although, the amount of collagen type III in the submucosa was still higher than in the normal tissue.

α -Smooth muscle actin. α -Smooth muscle actin staining was used to detect myofibroblasts. In normal unwounded palatal mucosa, α -smooth muscle actin was almost exclusively found around blood vessels. Myofibroblasts were found in considerable numbers in all wounds 1 week after surgery (Figure 3C). In general, there were more myofibroblasts in the deeper layers of the submucosa than just below the epithelium. In some samples, the myofibroblasts showed a focal clustering. At 3 weeks after surgery, the number of myofibroblasts had decreased in all wounds (Table 1), and at 12 weeks after surgery, the staining for α -smooth muscle actin was comparable with that of normal tissue (Figure 3D).

Leucocyte protein L1. Leucocyte protein L1 staining was used to detect neutrophils, monocytes, and macrophages and is also known to stain mucosal epithelium (Brandtzaeg *et al.*, 1987). In normal palatal mucosa, L1 was hardly expressed in the dermis. At 1 week after surgery, L1-positive cells were found in considerable numbers in all wounds (Figure 3E). At 3 weeks after surgery, only a few L1-positive cells remained in the wounds, and at 12 weeks after surgery, L1-positive cells were not detected in any of the samples within the wounds (Figure 3F and Table 1).

Discussion

The overall aim of this research was to develop an implantable mucosal substitute for CP surgery. The use of an autologous skin graft in a surgically created palatal defect by Jonsson and Hallmans (1980) showed a positive effect. Therefore, the implantation of a cultured substitute might

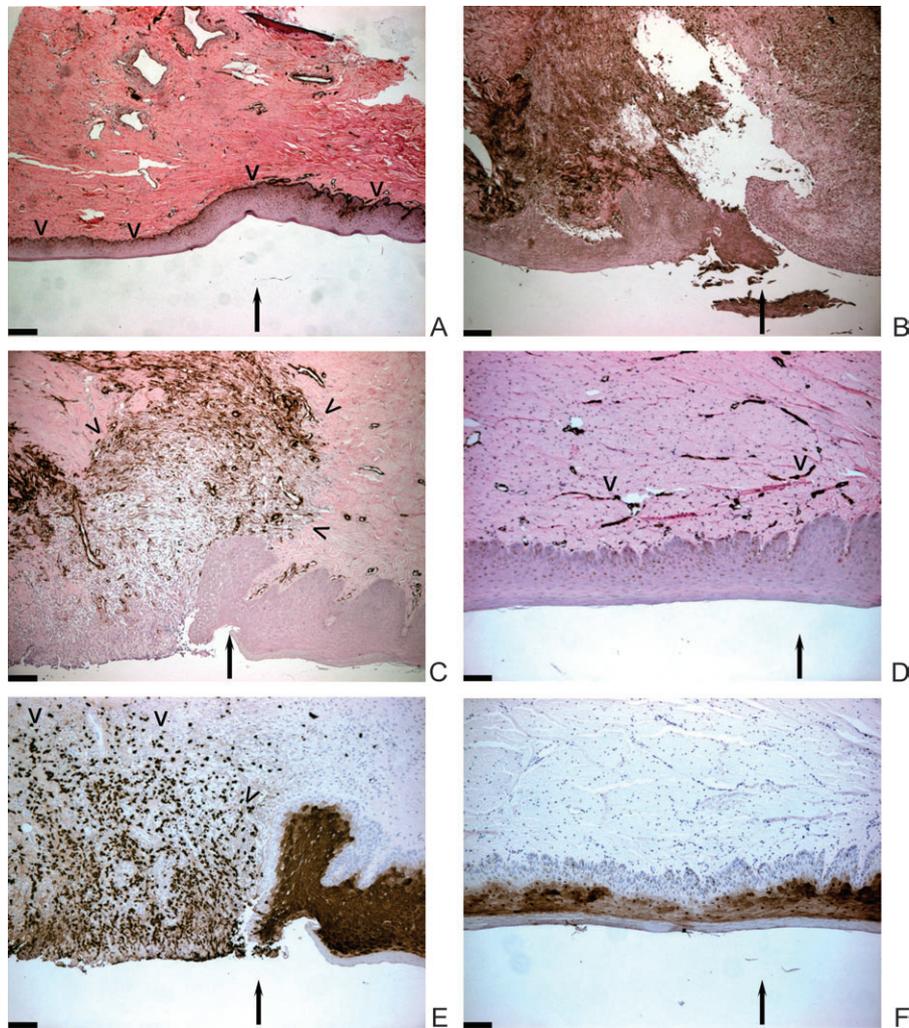


Figure 3 Immunohistochemistry of experimental wounds. (A) Heparan sulphate in a wound with the cultured oral substitute (AlloDerm®) 12 weeks after surgery. (B) Collagen type III in a wound with an autologous implant at 1 week after surgery. (C) α -Smooth muscle actin in an untreated wound 1 week after surgery. (D) α -Smooth muscle actin in an untreated wound 12 weeks after surgery. (E) Leucocyte protein L1 in a wound with a cultured oral substitute (de-epidermized dog dermis) 1 week after surgery. (F) Leucocyte protein L1 in a wound with a cultured oral substitute (AlloDerm®) 12 weeks after surgery. The specific proteins are stained brown and indicated by arrowheads (V) and the arrow (\uparrow) indicates the wound margin; the wound is left of this arrow. Bar = 50 μ m.

reduce wound contraction and scar tissue formation and prevent the inhibition of maxillary growth after palatal surgery. The aim of this specific study was to investigate the *in vivo* behaviour of oral mucosal substitutes composed of autologous oral keratinocytes cultured on two types of skin-derived substrates after implantation in open palatal wounds in the beagle dog.

The cultured mucosal substitutes showed histological and immunohistochemical characteristics close to normal oral epithelium, which was also found in a previous study (Ophof *et al.*, 2002). Therefore, it was hypothesized that such substitute may prevent the iatrogenic effects of CP repair.

The current findings showed, however, that the keratinocytes of the implants disappeared within the first

week after implantation. Moreover, the epithelium of the autologous biopsy had also disappeared at that time. Others have reported the clinical survival and even the outgrowth of keratinocytes from a sheet (Raghoobar *et al.*, 1995; Lauer and Schimming, 2001). However, in those studies, epithelialization was only examined macroscopically and not histologically. Thus, it is difficult to determine whether the epithelium originated from the sheet or proliferated and migrated from the wound margins. In the present study, epithelium from the wound margins had started to proliferate and migrate at 1 week post-surgery, when the epithelium had already been lost from the implant. This observed loss of epithelium is in agreement with previous reports on the healing of free gingival grafts placed

on periosteum (Brackett and Gargiulo, 1970; Caffesse *et al.*, 1979). Those studies also showed loss of the epithelium and degeneration of the graft. When the free gingival grafts were placed directly on bone, epithelial coverage was only restored after 14 days by outgrowth from the wound margins, which is in agreement with the present findings. It has been speculated that graft degeneration and delayed epithelialization are caused by the slow revascularization of the dermal component (Boyce *et al.*, 1995; Sumi *et al.*, 1999). The substitutes used in the present research were composed of keratinocytes and a cell-free dermal component. They were implanted directly on the palatal bone, and therefore, nutrient supply is only possible by diffusion from the lateral wound margins until neo-angiogenesis has taken place. This process might be stimulated by vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF, Nomi *et al.*, 2002; Hojo *et al.*, 2003).

In the present study, all substrates induced an inflammatory response and all matrices were quickly degraded or sequestered. Turnbull and Stross (1983) reported that their porcine skin-derived substrate showed no incorporation or vascularization after implantation in an open wound in the hamster cheek pouch. These findings suggest that all substrates show significant degeneration and epithelial migration underneath the material if applied in open wounds. Owens and Yukna (2001), however, implanted AlloDerm® in the submucosal layer of the palate of beagle dogs. In that situation, only a slight degradation of the substrate without signs of inflammation was found 1 month after implantation. This indicates that mucosal coverage might be essential for the nutrient supply of the implant material and to prevent contamination with bacteria, food, and debris.

A basal membrane was present in all cultured substitutes before implantation. However, it was not found in any of the wounds before 12 weeks after surgery, although these samples exhibited a complete epithelial regeneration from 3 weeks onwards. It is likely that not only the epithelium had disappeared after implantation but also the basal membrane, and that the formation of a new basal membrane only starts when the keratinocytes migrate from the wound margins over the granulation tissue. In another study, using the same antibody, it was reported that in wounded human skin heparan sulphate was absent for up to 2 weeks (Andriessen *et al.*, 1997). They speculated that the expression of this specific epitope was below the detection level in paraffin-embedded tissue.

Conclusions

Cultured autologous epithelium on a skin-derived substrate implanted as an oral mucosal substitute in palatal wounds in dogs has serious drawbacks. The epithelium is exfoliated after 1 week, and the dermal part of the substitute shows severe degradation. Since this might be related to a slow vascularization of the dermal substrate, future research

should aim at the stimulation of revascularization using growth factors such as VEGF or bFGF, which might improve the integration and survival of the substitute.

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