

Histologic evaluation of skin-derived and collagen-based substrates implanted in palatal wounds

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Tissue shortage complicates the surgery of cleft lip and palate anomalies and the healing of defects on the palate impairs growth of the dento-alveolar complex due to scar tissue formation. Implantation of substitutes into the wound area might overcome this adverse effect. The aim of this study was to compare the tissue response to three collagen-based (collagen type I substrate alone, or collagen coated with elastin or chondroitin-6-sulfate) and two skin-derived substrates (unprocessed dermis and AlloDerm) after implantation into 12 dogs. Histology was performed at 3, 10, and 20 days postsurgery. We showed that all substrates were well tolerated. However, it is unclear whether AlloDerm was rapidly degraded or if it was sequestrated. There was no elastin or collagen present in these wounds. All collagen-based substrates showed good epithelial regeneration, although heparan sulfate (JM 403) was absent. Wounds treated with the collagen-based substrates contained fewer myofibroblasts at 20 days postsurgery and the type III collagen fibers in the immature scar tissue were more randomly oriented than in an untreated wound. In conclusion, palatal wounds with a dermal substrate heal with fewer indications of scar tissue formation and evoke only a mild inflammatory reaction, which is preferred over the tissue reaction in an untreated wound. (**WOUND REP REG 2004;12:528-538**)

Oral mucosa defects are often an inevitable consequence of maxillofacial surgery. The surface area of these wounds is reduced by the proliferation and migration of cells as well as by wound contraction and scarring. When wound contraction and scar tissue formation occur during skeletal growth, it is proposed that it counteracts normal development of the facial skeleton. This occurs in cleft palate repair when primary surgery is performed on a growing child. During closure of the cleft, mucosal defects are produced on the lateral areas of the palate. Immediately after sur-

DED	De-epidermized dog dermis
H&E	Hematoxylin & eosin
PBS	Phosphate buffered saline solution

gery, maxillary development is inhibited by wound contraction. Later on, extensive scar tissue is formed, which is firmly anchored to the palatal bone. It has been strongly suggested that this scar tissue is a major factor in the impairment of skeletal growth and development of the dentition in both cleft palate patients and animal models.¹⁻³

Myofibroblasts are probably involved in wound contraction.^{4,5} These contractile fibroblasts are numerous in many types of contracting wounds. Subsequently, the myofibroblasts disappear, probably through apoptosis, and wound contraction ceases.⁵ Therefore, reducing the number of myofibroblasts during palatal wound healing after closure of the cleft might decrease wound contraction.

Histologic studies have shown that the tissue that develops by wound healing on the palatal bone after palatal repair in animal models is different from normal palatal mucosa. It lacks elastic fibers, the collagen

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fibers are oriented in a transverse direction, and the tissue is attached to the underlining bone by Sharpey's fibers.^{1,6} Therefore, this tissue is true scar tissue. It has been suggested that prevention of the attachment of scar tissue to the palatal bone might lead to a more favorable maxillary growth.¹

Contraction by myofibroblasts and scarring could possibly be reduced by the use of some graft material. Literature describes the transplantation of tissue from the radial forearm, pectoralis major, or free tissue flaps with microvascular anastomoses to reconstruct large defects.⁷⁻¹⁰ Although these techniques may be used successfully, additional problems may arise when the graft becomes necrotic or dysplastic.¹¹⁻¹³ Other problems may occur when the graft tissue maintains its original characteristics. Hair growth after transplantation of skin within the oral cavity has been shown in animal and clinical studies.^{14,15} To overcome these problems, the use of oral mucosal grafts from the buccal mucosa or the hard palate is described for the reconstruction of small defects in the oral cavity.¹⁶ However, it is difficult to obtain enough oral mucosa for the reconstruction of large defects.

To increase the availability of epithelium, in vitro culture techniques for keratinocytes have been used.¹⁷ However, an epithelium composed of cultured keratinocytes without a dermal substrate is very fragile, difficult to handle, and when grafted on a full-thickness skin wound, contraction of the connective tissue can still occur.^{18,19} When cultured keratinocytes are used in combination with a dermal substrate, re-epithelialization might be faster, wound contraction might be limited, and the formation of scar tissue might also be minimized.^{20,21} The most widely used types of dermal substrates are collagen-based and skin-derived materials. The collagen-based substrates generally consist of a type I collagen matrix sometimes supplemented with other extracellular matrix components such as elastin or glycosaminoglycans.^{22,23} The skin-derived substrates are prepared from glycerol-preserved cadaveric skin or processed to remove cellular components.²⁴

Dermal substrates consisting of type I collagen have been widely studied for the treatment of partial or full-thickness skin wounds. These substrates with or without autologous human keratinocytes and/or fibroblasts may have positive effects in the treatment of skin wounds in human and animal models.^{25,26} Collagen is biodegradable and exhibits little antigenicity.^{27,28} The formation of new dermis has been reported using a type I collagen substrate supplemented with chondroitin-6-sulfate glycosaminoglycans.²⁹ Dermal substrates derived from glycerol-preserved skin seem to have only limited value in treating full-thickness skin wounds because of rejection and the risk of viral transmission. These substrates may induce a stronger inflammatory response than the processed cadaveric

skin substrates because of remaining cellular components. Specifically, the processed skin-derived substrates were shown to be only weakly immunogenic or even nonimmunogenic and allowed host fibroblast infiltration, neovascularization, and keratinocyte migration.^{30,31} Processed skin-derived dermal substrates, like AlloDerm (LifeCell Corporation, Branchburg, NJ) have been used in clinical trials for burn wound management and soft tissue augmentation.^{32,33} They seem to function as a permanent dermal substitute and enhance wound healing.^{34,35}

In order to study the iatrogenic effects of palatal repair on skeletal growth and dental development, we conducted several experiments in which soft tissue palatal repair was performed in a beagle dog model.¹ In this study, it was suggested that the prevention of scar tissue attachment to the palatal bone leads to a more favorable skeletal growth. In follow-up studies, attempts were made to prevent the attachment of scar tissue by modifying the surgical techniques or by the use of biodegradable membranes. However, both approaches did not produce optimal long-term results.^{36,37}

The overall aim of our current studies is to develop a cultured substitute for oral mucosa containing autologous keratinocytes and a dermal substrate and to reduce the problem of contraction and scarring after cleft palate surgery. The specific aim of the present study was to evaluate the tissue response to several collagen-based and skin-derived substrates after implantation in palatal wounds in beagle dogs. Therefore, we have evaluated the inflammatory response, the presence of myofibroblasts, re-epithelialization, and the formation of a basement membrane. In addition, we evaluated the formation of scar tissue in the palatal mucosa. In a follow-up study, the most promising materials will be used as a substrate for keratinocyte culture and subsequent implantation in experimental wounds on the palate of beagle dogs.

MATERIALS AND METHODS

In this experiment, defects in the palatal mucosa of beagle dogs were filled with collagen-based substrates, skin-derived substrates, autologous mucosal implants or they were left open as a control. To evaluate the tissue response to the substrates, histology was performed at 3, 10, and 20 days postsurgery.

A total of 12 beagle dogs (age 2.5–3 years) were used in this study. 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.

Dermal substrates

Five different dermal substrates were used; unprocessed de-epidermized dog dermis (DED), commercially available processed human donor dermis (AlloDerm, LifeCell Corporation),³⁸ type I collagen substrate (kindly provided by Dr A. van Kuppevelt, Department of Biochemistry, University Medical Center Nijmegen, The Netherlands),³⁹ a type I collagen substrate coated with 3 percent w/w α -elastin hydrolysate (kindly donated by Dr E. Middelkoop, Burn Center, Department of Surgery, Red Cross Hospital, Beverwijk, The Netherlands),⁴⁰ and a type I collagen substrate with chondroitin-6-sulfate (IntegraTM, Medeco B.V., Oud-Beijerland, The Netherlands).⁴¹

DED was produced from full-thickness skin explants, as described previously for human skin.⁴² 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 solution (PBS) supplemented with 100 U/ml penicillin G and 100 μ g/ml streptomycin (Life Technologies, Breda, The Netherlands) at 37°C for two weeks to detach the epithelium. The DED was stored in PBS with antibiotics at -20°C. Before each experiment, the DED was thawed and washed three times in PBS. The silicon sheet covering the type I collagen/chondroitin-6-sulfate substrate was removed before the experiment. All substrates were washed three times in PBS before every experiment.

Surgical procedure

Prior to surgery, the animals were premedicated with 0.5 ml Thalamonal (0.05 mg fentanyl + 2.5 mg/ml droperidol; Janssen Pharmaceutica, Beersen, Belgium) and 0.5 ml atropine i.m. (0.5 mg/ml atropine sulfate). Subsequently, they were anesthetized with an intravenous injection of 30 mg/kg Narcovet (60 mg/ml sodium pentobarbital; Apharmo, Arnhem, The Netherlands). After intubation, anesthesia was maintained with Ethrane (15 mg/mg enflurane; Abott, Amstelveen, The Netherlands). The palatal mucosa was cleaned with Betadine solution (povidoniodine; Dagra-Pharma, Diemen, The Netherlands). In addition, approximately 2 ml Xylocaine (0.4 mg/ml lidocaine-hydrochloride + 0.0125 mg/ml epinephrine; Astra Chemicals, Rijswijk, The Netherlands) was injected into the palatal mucosa to avoid excessive bleeding during the procedure.

The 12 dogs were randomly assigned to three groups before surgery. The three groups of four dogs were evaluated at 3, 10, and 20 days postsurgery. In all dogs, four to six standardized full-thickness wounds were created on the palate with a 4-mm biopsy punch. The circular soft tissue defects were made between the

rugae at both sides of the palate. One randomly chosen wound served as a control and was left open. A second randomly chosen wound was filled with an autologous mucosal punch biopsy from one of the other wounds. The other biopsy wounds were randomly filled with one of the five experimental dermal substrates. To prevent loss of the substrate, every substrate was fixed gently in place using two sutures (4-0 Vicryl, Ethicon; Johnson & Johnson Company, Amersfoort, The Netherlands). All five substrates, the control wound, and the autologous implant were evaluated in triplicate at each time point. All animals received a normal diet after surgery.

Histologic procedures

At the time of euthanasia, the animals were brought under general anesthesia using 30 mg/kg Narcovet. After several minutes, a lethal dose of Narcovet was injected intravenously. The maxillae were dissected and immersed in 4 percent paraformaldehyde in 0.1 M phosphate buffer at room temperature. After fixation, the maxillae were sawn into six smaller blocks, each containing a biopsy wound. The tissue blocks were decalcified in 20 percent formic acid and 5 percent sodium citrate. Decalcification was checked radiographically. Palatal mucosa samples taken outside the wounded areas served as normal controls. Next, the tissue was dehydrated through a graded series of ethanol solutions and embedded in paraffin. Serial paraffin sections of 7 μ m were made through the entire wound. Each tenth section was mounted onto a superfrost (Menzel-Gläser, Braunschweig, Germany) slide and stained with hematoxylin & eosin (H&E) for general tissue survey. For further histologic analyses, six consecutive sections from the center of each wound were taken. Elastin was detected with the Weigert-Van Gieson staining and Sirius red staining was used to visualize the collagen fibers.^{43,44} The presence or absence of the substrate in each specimen was determined on sections stained with Weigert-Van Gieson. When elastic fibers were found in the wound area, we concluded that the substrate was present because those fibers are found in all skin-derived substrates and the type I collagen substrate coated with three percent w/w α -elastin hydrolysate and are not present in control wound tissue. The wound area of each specimen was blindly evaluated by one of the authors (RO) for the degree of inflammation and epithelial regeneration using H&E stained sections and the orientation and amount of elastic fibers (Weigert-Van Gieson staining). The scoring system used for degree of inflammation and the amount of the elastic fibers was on a scale of 0 (none) to 4 (abundant). Epithelial regeneration was given a score from 0 to 4 (0 = no cell layers; 1 = closed with some cell layers (<10); 2 = closed with many cell layers and no rete peg formation; 3 = closed with many cell

layers and some rete peg formation; 4=closed with many cell layers and normal rete peg formation). A mean value of the three sections of each time point was used.

Immunohistochemical procedures

Immunohistochemical analysis was performed on native palatal mucosal tissue and the 3, 10, and 20 days postsurgical sections to detect heparan sulfate, type III collagen, and α -smooth muscle actin. The paraffin sections were collected on Superfrost Plus slides (Menzel-Gläser), deparaffinized, and rehydrated again. Before staining, the slides were treated with 0.1 percent trypsin 250 (DIFCO Laboratories, Detroit, MI) for 10 minutes and rinsed with PBS. Thereafter, the slides were treated with 3 percent H_2O_2 in PBS for 30 minutes to block endogenous peroxidase and rinsed in PBS.

The anti-heparan sulfate JM 403 antibody (mouse antihuman heparan sulfate, IgM) was a kind gift of Prof. Dr J. Berden (Department of Nephrology, University Medical Center, Nijmegen, The Netherlands).⁴⁵ The sections were preincubated with 5 percent bovine serum albumin in PBS buffer (BSA, Sigma-Aldrich, St. Louis, MO). After pretreatment, the sections were incubated with mouse anti-heparan sulfate IgM for one night at 4°C. After washing with PBS, the sections were incubated with a biotinylated goat anti-mouse antibody 1:400 (Vector Laboratories, Burlingame, CA) for 60 minutes at room temperature. After washing with PBS, the sections were treated with ABC-peroxidase (Vector Laboratories). Peroxidase activity was visualized with a standard diaminobenzidine technique (Sigma-Aldrich). The sections were counterstained with Dellafields hematoxylin.

Type III collagen was detected with a rabbit anti-collagen III antibody (Chemicon, Temecula, CA). The staining method was the same as described for heparan sulfate except the secondary antibody was a biotinylated goat anti-mouse antibody (Chemicon).

α -smooth muscle actin was detected by a mouse anti-smooth muscle actin antibody (1:1600) (Sigma-Aldrich). The staining method was the same as described for heparan sulfate. Only the secondary antibody was a biotinylated donkey anti-mouse antibody (1:500; Jackson Laboratories, West Grove, PA).

The presence or absence of heparan sulfate in each specimen was determined. The scoring system used for the presence of type III collagen and α -smooth muscle actin was on a scale of Ø (none) to 4 (abundant). Mean values of the three sections of each time point were used. Representative sections were photographed on a Leitz DMRD microscope (Leica, Wetzlar, Germany).

RESULTS

The palatal mucoperiosteum of a beagle dog consists of four layers: the epithelium, the lamina propria, the submucosa, and the periosteum (Figure 1A). The palatal epithelium contains a basal layer with cubical and columnar cells. Superficial to the basal layer, on average 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 retained. The lamina propria contains a relatively loose network of collagen fibers while in the deeper layers more densely packed collagen fibers 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 periostial part of the mucoperiosteum consists of two layers, a fibrous layer and a thin layer with some osteoblasts. Only a few fibers connect the fibrous layer of the periosteum to the palatal bone.

Histology of experimental wounds

At three days postsurgery, a blood clot and many polymorphonuclear leukocytes were present in the untreated wounds (Figure 1B). The epithelium had already started to proliferate and had migrated from the wound edges under the blood clot. None of the untreated wounds was closed at this time. The wounds were filled with granulation tissue that was rich in fibroblasts, granulocytes, and an extracellular matrix without a clear organization. Neither elastin nor collagen fibers were observed in the wounds.

The implants could easily be recognized in the wounds filled with an autologous implant (not shown). Remaining keratinocytes with large vacuoles were present at three days postsurgery. Below the implant, the tissue organization was similar to that of the untreated wounds at three days. Elastin and collagen staining was clearly observed in the autologous mucosal implants. The collagen fibers were thick and resembled that of normal palatal mucosa. A noncollagenous tissue was surrounding the implant.

The wounds filled with the skin-derived substrates showed histologic characteristics different from the autologous ones. DED was easily detectable in the wounds (Figure 1C), except for one sample. Elastin staining was always present in the DED and thick collagen fibers were detected with Sirius Red staining (Figure 1D). A noncollagenous tissue surrounded the substrate. In contrast, in all wounds filled with Allo-Derm, there was no evidence that the substrate was still present in the wound area (Figure 1E). This finding was supported by the fact that no elastin or collagen was present in the wound area.

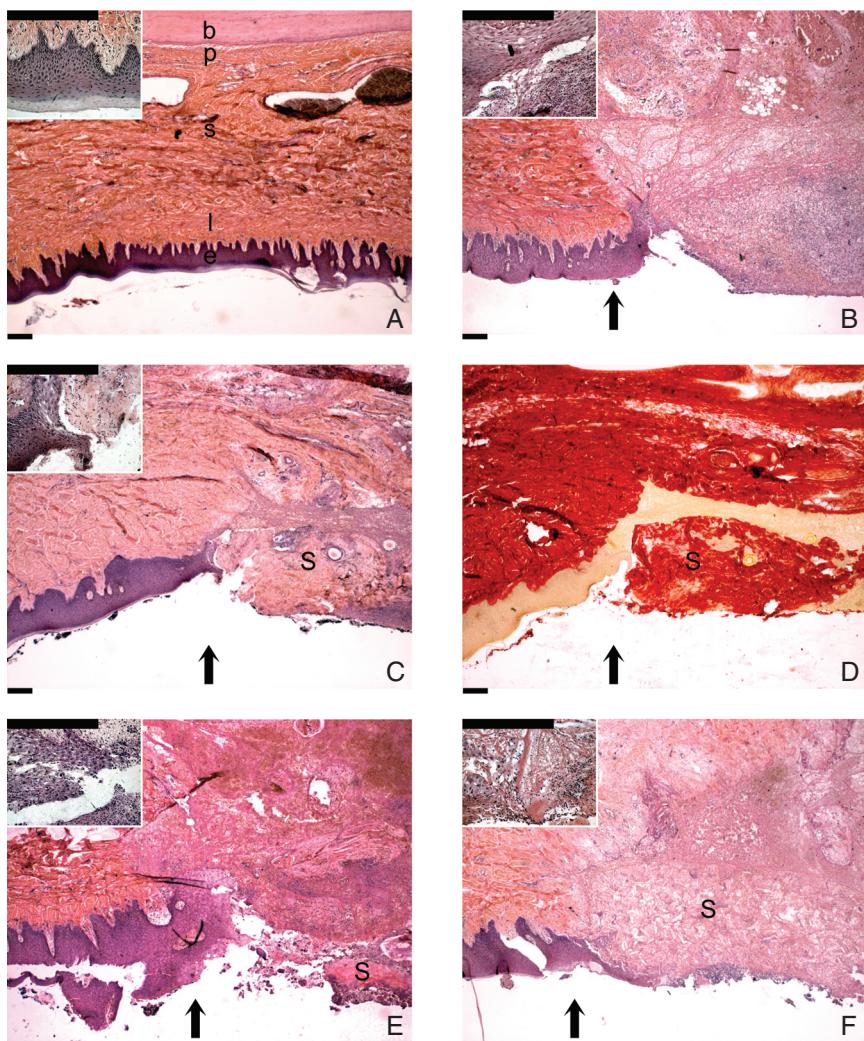


FIGURE 1. Histology of normal palatal mucosa and experimental wounds at three days postsurgery. (A) Normal palatal mucosa of a beagle dog, (B) untreated wound at three days postsurgery, (C) skin-derived substrate DED at three days postsurgery, (D) skin-derived substrate DED at three days postsurgery (Sirius RED), (E) skin-derived substrate AlloDerm at three days postsurgery, (F) collagen-based substrate Integra at three days postsurgery. All sections are H&E stained except if noted. S = substrate, e = epithelium, l = lamina propria, s = submucosa, p = periosteum, b = bone and ↑ is wound margin. The bar represents 50 μm. (Original magnification 50x).

The collagen-based substrates were easily recognized in the wounds, especially Integra because of its characteristic structure (Figure 1F). The inflammatory response and the epithelial regeneration for both skin-derived substrates and the collagen-based substrates were comparable to that of the untreated wounds (Table 1).

At 10 days postsurgery, the untreated wounds were covered with up to five epithelial cell layers but rete pegs had not yet formed (Figure 2A). The wound was completely filled with granulation tissue that was rich in fibroblasts, granulocytes, some blood vessels, and thin collagen fibers. The inflammatory infiltrate was still present, although it was reduced compared to three days postsurgery. The collagen fibers had a mainly transverse orientation. Superficial osteoclastic bone resorption was found in all samples.

The histologic findings of the autologous implants (Figure 2B) and both skin-derived substrates were comparable to the untreated wounds. However, the epithelium covering both skin-derived substrates contained more cell layers than the untreated wounds and

some signs of rete peg formation were already visible. The skin-derived substrates AlloDerm and DED were difficult to identify in four of the six samples because of severe degradation. In the remaining samples, no AlloDerm or DED was found within the wound area. These samples also failed to show elastin staining which indicates the absence of these substrates. The inflammatory response was predominantly chronic in all of the wounds except for one of the wounds filled with AlloDerm in which an acute inflammatory reaction with many polymorphonuclear leukocytes was still visible. Thick collagenous bundles with a random orientation were observed in the autologous implants and the DED remaining in the wounds. In all wounds, thin collagen fibers with a parallel orientation were enclosing the substrates if present. In the wounds filled with the autologous mucosal implants and the skin-derived substrates, elastin staining was present in remaining patches of the substrate.

The collagen-based substrates could still be easily recognized, especially Integra, because of its characteristic structure. Thin collagen fibers were aligned in

Table 1. Histologic changes in the experimental wounds*

Treatment group-parameter	Time (d)		
	3	10	20
Untreated wounds			
Epithelial regeneration	0	1	2
Inflammation	3	2	1
Elastic fibers	0	0	0
Autologous mucosal grafts			
Epithelial regeneration	0	1	2
Inflammation	3	2	1
Elastic fibers	2	1	1
Skin-derived substrate AlloDermR			
Epithelial regeneration	0	1	2
Inflammation	3	3	1
Elastic fibers	0	1	0
Skin-derived substrate DED			
Epithelial regeneration	0	1	3
Inflammation	3	2	1
Elastic fibers	2	1	1
Collagen-based substrates			
Epithelial regeneration	0	1	3
Inflammation	3	2	1
Elastic fibers	0	0	0

*Epithelial regeneration was given a mean absolute score from 0 to 4 (0 = no cell layers; 1 = closed with some cell layers [< 10]; 2 = closed with many cell layers no rete peg formation; 3 = closed with many cell layers and signs of rete peg formation; 4 = closed with many cell layers and rete peg formation). The mean absolute values for inflammation and elastic fibers represent 0 = none to 4 = abundant.

transverse direction and seemed to enclose these substrates (Figure 2C). The inflammatory infiltrate outside the implant was comparable to the untreated wounds although in some samples there were signs of a somewhat patchy infiltrate of inflammatory cells within the substrate (Table 1).

At 20 days postsurgery, the epithelium had become thicker in the untreated wounds and a cornified layer had formed, although rete pegs had not yet developed (Figure 2D). The inflammatory infiltrate had decreased but some inflammatory cells were still present in the wounds (Table 1). The number of fibroblasts had also decreased. Bone apposition was found at some sites where the bone probably has been damaged by the biopsy punch.

The tissue organization of the wounds filled with an autologous implant was in general comparable to that of the untreated wounds at 20 days (not shown). However, the epithelium contained more cell layers and the thick collagenous bundles of the original implant were still present as shown by the elastin staining.

The wounds filled with skin-derived substrate AlloDerm were different than the wounds filled with DED. AlloDerm could not be detected in two of the three samples, and in these two samples, there was also no elastin staining present within the wound area. In contrast, elastin staining was always present in the wounds filled with DED (Figure 2E) and the histologic findings were comparable to that of the wounds with an autologous implant. However, the epithelium covering the

wounds filled with DED consisted of more cell layers than the wounds with the autologous implants, and more pronounced rete pegs were present (Figure 2F). The orientation of the collagenous bundles in the superficial layer of the dermis was more random with less transverse thick bundles. However, in the deeper layers of the wound, they were oriented in a transverse direction with more dense collagenous bundles. These parallel collagen fibers were surrounding an area containing collagen fibers with a random orientation.

The number of cell layers in the epithelium covering the collagen-based substrates was not increased compared to 10 days postsurgery, but rete peg formation was visible (not shown). The substrates contained many fibroblasts. Collagenous bundles with a transverse orientation had become thicker and had enclosed the substrate. There were still no signs of substrate degradation, which was comparable to three days postsurgery (not shown). In some samples, there was a mild inflammatory infiltrate still present at some spots within the substrate (Table 1). However, in these samples, the inflammatory response outside the substrate was comparable to an untreated wound.

Immunohistochemical findings

Heparan sulfate staining was used to evaluate the formation of a basement membrane. Continuous staining for heparan sulfate was always observed at the epithelial-dermal border of the normal mucosa and only a few patches in the autologous mucosal grafts at three days postsurgery (not shown). Although in the end, all wounds were closed with a newly formed epithelium and some rete peg formation was also present in the wounds filled with the skin-derived DED and collagen-based substrates, there was no sign of heparan sulfate in any of the wounds at any time point (Figures 3A and Table 2). However, continuous staining for heparan sulfate was always present in all the samples away from the wound areas.

In the unwounded palatal mucosa, an intensely stained network of loose type III collagen fibers was present in the papillary layer of the submucosa, and around blood sinuses. The staining in the other regions of the submucosa was very weak.

Type III collagen was not present in any of the wounds at three days postsurgery. At 10 days postsurgery, all groups showed type III collagen staining which was more intense closer to the epithelium than deeper in the wounds (Figure 3B). The wounds filled with the collagen-based substrates also showed collagen III staining within the substrate and in the deeper layers of the submucosa between the substrate and the bone.

Twenty days after surgery, the intensity of the type III collagen staining was reduced in all wounds. In the center of the collagen-based substrates, staining for

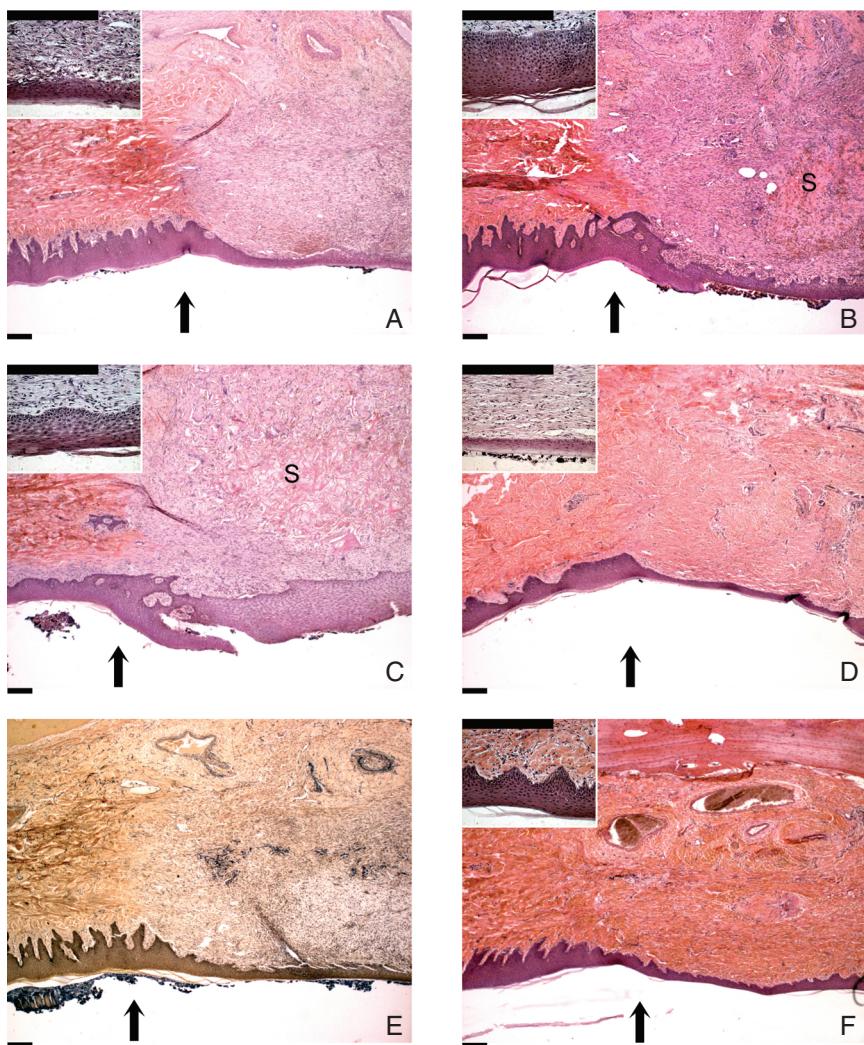


FIGURE 2. Histology of experimental wounds at 10 and 20 days postsurgery. (A) Untreated wound at 10 days postsurgery, (B) autologous implant at 10 days postsurgery, (C) collagen-based substrate Integra at 10 days postsurgery, (D) untreated wound at 20 days postsurgery, (E) skin-derived substrate DED at 20 days postsurgery (elastin staining), (F) skin-derived substrate DED at 20 days postsurgery. All sections are H&E except if noted. S=substrate and ↑ is wound margin. The bar represents 50 μm. (Original magnification 50×).

type III collagen was also reduced compared to 10 days postsurgery (Figure 3C).

α -smooth muscle actin staining was used to detect myofibroblasts. In normal unwounded palatal mucosa, α -smooth muscle actin staining was only found around the blood vessels.

In none of the wounds α -smooth muscle actin staining was found at three days postsurgery (not shown). Positive cells were found in all wounds at day 10 in considerable numbers (Figures 3D and Table 2). In general, there were more α -smooth muscle actin positive cells directly below the epithelium than in the deeper layers of the submucosa. There was a less intense staining in the wounds with the skin-derived substrates compared to the open wounds. Within the collagen-based substrates, the staining was more intense in the deeper layer of the substrate and in the area between the substrate and the bone compared to all other wounds (not shown). The α -smooth muscle actin staining in some samples was locally more intense. However, the general distribution of positive cells was equal to the other wounds (Table 2).

At day 20, the amount of α -smooth muscle actin positive fibroblasts in all wounds had decreased compared to 10 days postsurgery (Table 2). However, some positive cells were still present in the granulation tissue. In the wounds with an autologous implant or filled with DED, some spots with an intense staining for α -smooth muscle actin were found. This area was surrounded with a layer of parallel collagen fibers without any α -smooth muscle actin positive fibroblasts (Figure 3E). Within the collagen-based substrates, myofibroblasts had disappeared at 20 days, but in a few samples, some positive cells were still present around the substrate (Figure 3F).

DISCUSSION

The overall aim of our research is to develop a cultured mucosal substitute for implantation on the palate after cleft palate surgery. The implantation of some graft material into an open wound might reduce wound contraction and scarring.³⁴ The aim of this specific

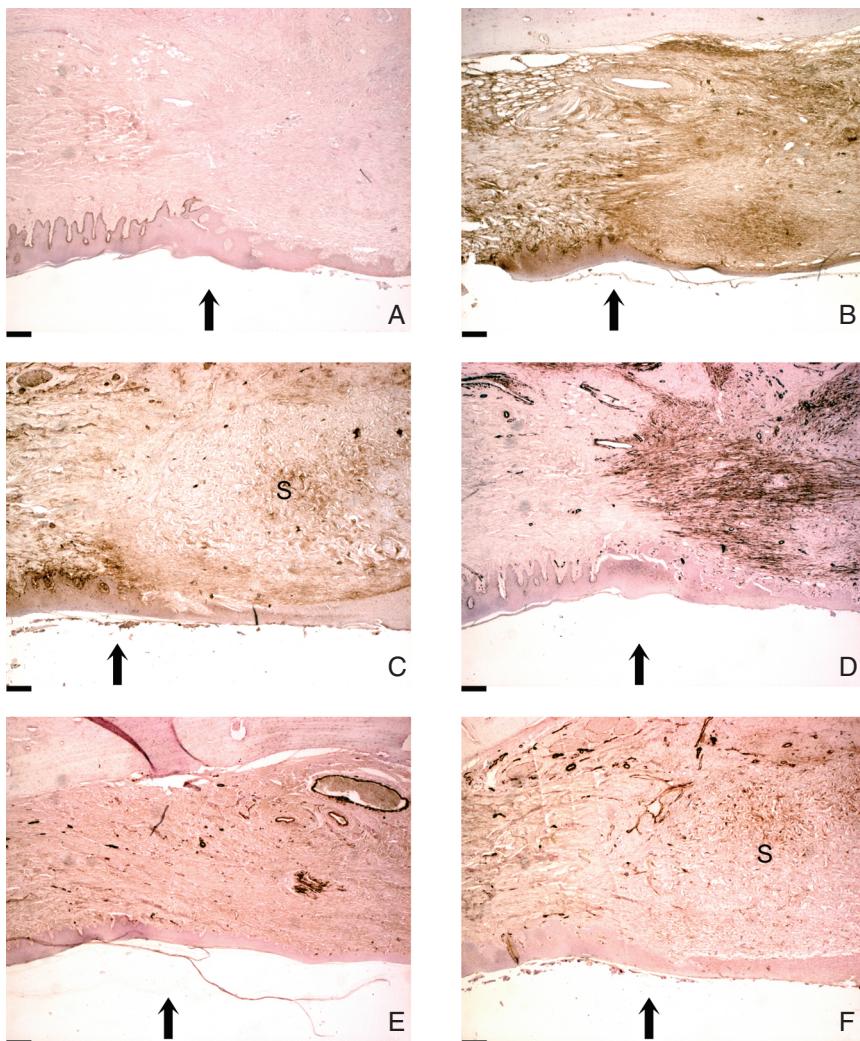


FIGURE 3. Immunohistochemistry of experimental wounds at 10 and 20 days postsurgery. (A) Heparan sulfate in a wound with DED at 20 days postsurgery, (B) type III collagen in an untreated wound at 10 days postsurgery, (C) type III collagen in a wound with Integra at 20 days postsurgery, (D) α -smooth muscle actin in an untreated wound at 10 days postsurgery, (E) α -smooth muscle actin in a wound with DED at 20 days postsurgery, (F) α -smooth muscle actin in a wound with Integra at 20 days postsurgery. S=substrate and \uparrow is wound margin. The bar represents 50 μ m. (Original magnification 50 \times).

Table 2. Immunohistochemical changes in the experimental wounds*

Treatment group-antigen	Time (d)		
	3	10	20
Untreated wounds			
Heparan sulfate	0	0	0
Collagen type III	0	2	1
α -Smooth muscle actin	0	2	1
Autologous mucosal grafts			
Heparan sulfate	1	0	0
Collagen type III	0	2	1
α -Smooth muscle actin	0	2	1
Skin-derived substrates			
Heparan sulfate	0	0	0
Collagen type III	0	2	1
α -Smooth muscle actin	0	2	1
Collagen-based substrates			
Heparan sulfate	0	0	0
Collagen type III	0	3	1
α -Smooth muscle actin	0	2	1

*The value for heparan sulfate represents 0 = absent and 1 = easily distinguishable. The scoring system used for the presence of type III collagen and α -smooth muscle actin was on a scale of 0 (none) to 4 (abundant).

study was to investigate the *in vivo* behavior of several dermal substrates after implantation in open palatal wounds in the beagle dog. Subsequently, in a follow-up study, the most promising dermal substrate will be used for keratinocyte culture and implantation in experimental wounds on the palate of beagle dogs.

In the histologic evaluation, the general tissue response of the palatal mucosa was studied and more specifically the substrate degradation, inflammation, epithelial regeneration, basement membrane formation, and collagen orientation.

The present study has shown that most of the substrates were hard to detect in the specimens, except for the collagen-based substrate Integra because of its characteristic structure. We used elastin staining as an aid to identify the substrates in the wound areas. The samples with an autologous implant or DED always showed the presence of elastic fibers. Because elastic fibers are not present in control wound tissue,

we concluded that both substrates were still present in the wounds. On the other hand, none of the samples with the collagen-based substrates and only few with AlloDerm showed elastin fibers. Stricklin and Nanney⁴⁶ reported that in the early stages of wound healing degradative processes within the wound bed take place. Lamme et al.⁴⁷ also showed that elastic fibers remained undetectable up to three weeks after implantation, although they implanted a collagen type I substrate coated with elastin. Therefore, it might be possible that the elastin present in those substrates was rapidly degraded after implantation. Another possibility might be that the collagen-based substrates and AlloDerm were sequestered by the epithelium that grew under it.

All substrates showed severe degradation starting 10 days postsurgery except for Integra that did not show any signs of degradation up to 20 days postsurgery. Kremer et al.⁴¹ also reported that Integra grafted in an athymic mouse showed only fibroblast and endothelial cell ingrowth without any signs of substrate degradation up to 15 days postsurgery. At eight weeks postsurgery, they found that the Integra was completely replaced by native connective tissue. These findings indicate that Integra might have been replaced if a longer follow-up period was included in our study.

The present study has further shown that all substrates were well tolerated by the oral mucosa except for AlloDerm. Others^{48,49} have shown that the rejection of such a dermal substrate is due to a specific immune response to its cellular components. As AlloDerm is supposed to be cell-free, it is unlikely that the low survival of the AlloDerm is due to a specific immune response. The findings of our study are not in agreement with those of Owens and Yukna⁵⁰ who also implanted AlloDerm in the palate of beagle dogs. They showed a slight to moderate degradation of the substrate without signs of inflammation at one month after implantation, and a severe to complete degradation of the substrate at four months after surgery. However, they used AlloDerm as a subcutaneous implant, and not, like in our study, in an open wound. It would be reasonable to assume that the tissue reaction to the substrate is different when there is a mucosal coverage. Turnbull and Stross⁵¹ reported that their porcine skin-derived substrate showed no incorporation or vascularization after implantation in an open wound in the hamster cheek pouch within 28 days postsurgery. Although the animal model and the implantation site are different from ours, their findings support ours. This could be explained by the use of open wounds and subsequent wound healing by secondary intention in both models. In general, it might be concluded that all substrates were well tolerated by the oral mucosa, but it seems that AlloDerm is not incorporated well.

In none of the wounds with DED or the collagen-based substrates heparan sulfate was detected, although these samples exhibited the most complete epithelial regeneration. Heparan sulfate remained undetectable during the entire experimental period, which indicates that in spite of an intact epithelium, the basement membrane was not yet completely restored. Andriessen et al.,⁵² using the same antibody, reported that in wounded human skin, heparan sulfate was absent for up to two weeks. They speculated that in that time range the expression of this specific epitope was still below the detection level in paraffin-embedded tissue.

The untreated wounds in our study showed parallel collagen fibers and considerable numbers of myofibroblasts at 10 days postsurgery, which is in agreement with others. It has been shown that the collagen fibers in a healing wound tend to run in the direction of the contraction forces.⁵³ Squier and Kremenak⁴ have shown that myofibroblasts are present in healing palatal wounds of the beagle dog from 7 days postsurgery, and are most numerous between 10 and 15 days. The wounds filled with a skin-derived substrate showed less myofibroblasts than an untreated wound at 10 days postsurgery. Furthermore, the collagen fibers in the wounds with DED no longer contained myofibroblasts at 20 days postsurgery. Also in the collagen fibers surrounding the collagen-based substrates, myofibroblasts were not encountered. Myofibroblasts had disappeared within these substrates from 20 days postsurgery. Hence, it seems clear that the collagen-based substrates showed fewer myofibroblasts than the untreated wounds. These findings indicate that both the skin-derived and the collagen-based substrates induce less wound contraction than occurs in an untreated wound.

Our results show that palatal wounds with an implant heal with fewer indications of scar tissue formation and evoke only a mild inflammatory reaction, which is preferred over the tissue reaction in an untreated wound. The collagen fibers in the immature scar tissue are more randomly oriented than in an untreated wound. The wounds filled with a skin-derived substrate, DED, or collagen-based substrates showed the best epithelial regeneration. In general, the wounds treated with these substrates showed less myofibroblasts compared to the untreated wounds. It is not completely clear whether the skin-derived substrate AlloDerm was rapidly degraded or that it was sequestered by the epithelium that grew under it. Based on the findings of this study, all substrates will be used for further studies on keratinocyte culture. Subsequently, the keratinocyte-seeded substrate with the best histologic characteristics will be implanted in experimental wounds on the palate of beagle dog. This may ultimately lead to the improvement of cleft palate surgery in humans.

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