CASE REPORT

Poor Biointegration of Porcine Acellular Dermal Matrix Associated with Unfavorable Gingival Healing: A Report of Three Cases

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ABSTRACT

Aim: The aim of the present work was to explain the poor biointegration of acellular dermal xenogeneic matrix, leading to an unfavorable gingival healing following a grafting procedure for the treatment of soft tissue deficiencies.

Background: Numerous works have demonstrated the successful use of acellular dermal matrix (ADM) in soft tissue augmentation procedures. However, spare human investigations reported adverse healing outcomes at microscopic level.

Case description: Three patients showing various soft tissue deficiencies (recession, gingival thickening) requiring a gingival augmentation were grafted using an ADM porcine acellular dermal matrices (pADM) as a soft tissue substitute. For this purpose, appropriate soft tissue augmentation surgeries were performed and the grafted pADM was left for proper healing. Biopsies were harvested from two out of the three patients, respectively, at 11 and 27 weeks in order to conduct a histological evaluation of the pADM's doubtful biointegration. Moreover, the ultrastructural analysis of pADM was performed using scanning electron microscopy, and additional histological procedures were used to assess its ability to support human gingival fibroblast cultures. Signs of gingival inflammation persisted several months postoperatively. Histologically, numerous inflammatory cells characterized the grafted site. Indeed, the high number of foreign body giant cell granulomas and the very densified newly formed collagen fibers highlighted a fibrotic process within gingival connective tissue.

The ultrastructural and histological analysis showed that pADM was characterized by very thick and dense collagen bundles demonstrating a nonphysiological collagen network organization. Cell culture experiments showed fibroblasts proliferating on the matrix surface, sparing its deeper part, even though the collagen matrix degradation seemed to occur following a gradient from the pADM surface inward.

Conclusion: The unfavorable clinical results may be caused by the poor colonization of matrix cells and poor angiogenesis leading to the inadequate biointegration of pADM. Hence, the pADM structure in terms of porosity and degradability should be further investigated.

Clinical significance: The present cases highlighted a poor integration of pADM following soft tissue grafting procedures, which was caused by the inadequate ultrastructure of the used pADM. Therefore, despite the utility of such tissue substitutes, their manufacturing improvement could be required to obtain a better biointegration.

Keywords: Acellular dermal matrix, Human gingival fibroblasts, Soft tissue augmentation.

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BACKGROUND

Soft tissue thickness is an important factor in the protection of both natural dentition and dental implants.¹ Teeth with soft tissue deficiencies cause substantial therapeutic problems, mainly pertaining to aesthetics and hypersensitivity;² on the other hand, a thick peri-implant gingiva plays a major role in reducing the prevalence of peri-implant diseases.³ Similarly, for fixed tooth-supported prosthesis with keratinized tissue ≥ 2 mm connective tissue grafts (CTGs) significantly increase the gingival lining,⁴ thus reducing plaque index, gingival inflammation and attachment loss, as compared to equivalent nongrafted sites.⁵ Moreover, it is well known that tooth extraction is accompanied by volumetric tissue changes, more than 60% of which occur in the first 3 months, thus creating a concavity on the buccal side.⁶

In order to treat such tissue defects, both connective or epithelial-CTGs are regarded nowadays as the gold standard.⁷ Over the years, various modifications to the original technique have been developed, but despite their highly predictable results, they still present numerous disadvantages, particularly an additional surgical site.⁸ To avoid these drawbacks, various alternative solutions have been advocated in the last 10 years,⁹ such as xenogeneic and

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alloplastic substitutes, which were proven to be a safe solution despite having lower success rates than autogenous grafts.¹⁰ Moreover, the use of ADM allografts may be associated with ethical concerns and the potential risk of disease transmission. Thus, as an alternative, xenogeneic acellularized materials were introduced.¹¹ Several clinical and preclinical studies reported their successful use for root coverage and soft tissue thickening procedures.^{12,13}

Because of its nonvital structure, ADM acts as a three-dimensional scaffold that depends on recipient site cells and blood vessels to achieve reorganization, causing a slower incorporation that could ultimately result in structural and functional impairment. This original graft matrix then degrades through new connective tissue production and eventually becomes completely replaced by host tissues.¹⁴ However, the efficiency of cell incorporation throughout the matrix is still under investigation as previous studies showed that pADM are only partially colonized *in vitro* by gingival fibroblasts. Thus, cells are preferentially located on the surface and unable to migrate from the periphery of the matrix inward.^{15,16}

As for autogenous grafts, ADM was proposed as an alternative for ridge augmentation, and soft tissue thickening¹⁷ to correct aesthetic and functional problems.¹⁸

In the present study we report adverse outcomes observed in three patients grafted with pADM to treat soft tissue deficiencies around teeth and implants. Due to these unsatisfactory clinical results, it became necessary to understand the dynamics of pADM

in situ biointegration. For this purpose, it was possible to observe histologically the biointegration of the current grafted pADM. Furthermore, in order to elucidate the histologically observed poor pADM biointegration, ultrastructural analyses, and *in vitro* studies using human gingival fibroblast cultures were also performed.

CASE DESCRIPTION

This study involved three patients showing soft tissue deficiencies who underwent soft tissue grafting surgery (at Saint-Joseph University Dental Clinics), using pADM as an alternative to connective tissue autogenous graft. Two out of three (Patients #1 and #2) presented gingival recessions resulting in tooth sensitivity and unaesthetic aspect while the third one (Patient #3) had a buccal horizontal bone defect impairing both the esthetic and functional results of the future implant site. We had the opportunity to harvest biopsies in two out of the three patients (Patients #2 and #3) without any modification of the surgical procedure.

Patient 1

The first patient was a 37-year-old male with a 2-mm gingival recession (Class 1 of Miller) on the first upper left premolar (Fig. 1A). In order to harmonize the gingival smile line and reduce hypersensitivity, a buccal recession on tooth 24 was grafted with a pADM then covered with a partial thickness coronally repositioned

Figs 1A to I: Clinical observations: Patients 1 (A to C), 2 (D to F), 3 (G to I). (A) Recession on 24; (B) Redness persists, 4 months following surgery; (C) At 1-year, redness persistence; (D) Gingival recession on 14, 15; (E) Day 0, partial-thickness tunnel flap; (F) Three months, total root coverage; (G) Restoration site; (H) pADM designed according to the buccal defect; (I) Six months after prosthetic restoration, unsuitable redness was still observed

flap.¹⁹ Following total root debridement, the membrane was thoroughly hydrated in a saline buffer then reshaped according to the grafted site and positioned apically to the cemento-enamel junction and stabilized with absorbable sutures. Four months following the pADM grafting procedure, a clinical examination of the surgical site revealed poor healing with persistence of redness corresponding to gingival inflammation that was painless yet firm upon palpation (Fig. 1B). At 1-year post-op, the color of the grafted area was still discordant with its surrounding gingiva, and the gum recession was reduced to 0.5 mm while the keratinized tissue thickness was 2–3 mm. Furthermore, palpation confirmed the persistence of a firm mass at the grafted site (Fig. 1C).

Patient 2

The second patient was a 33-year-old male complaining from tooth hypersensitivity caused by a gingival recession (Class 1 Miller) on the two upper right premolars (14 and 15) with no carious cervical lesions (Fig. 1D). The recessions were treated using a tunnel technique. Following the preparation of a partial-thickness flap mobilizing the marginal gingiva, a mucogingival tunnel was prepared into which the pADM membrane was introduced and stabilized by sutures (Fig. 1E).²⁰ At 3 months, a noticeable increase in the attached gingiva was observed with total root coverage of the second right premolar (Fig. 1F). However, the grafted site clinically showed a normal-looking attached gingiva in harmony with its surroundings. At 27 weeks, concomitantly with a homolateral wisdom tooth removal next to the grafted area, a biopsy was carried out within the grafted site for histological analysis of the pADM biointegration, with the patient's consent without modifying the original surgical procedure.

Patient 3

The third patient was a 35-year-old male who came in for the replacement of his edentulous first left upper premolar. Clinical examination revealed a thin biotype associated with a reduced amount of keratinized gingiva in both height and thickness (Fig. 1G). No periodontal pockets were revealed after probing. The treatment of choice was implant rehabilitation for conservative and functional reasons. The presence of a horizontal bone defect at the future implant site had to be treated to enhance the buccal contour. Then a pADM was hydrated in a saline buffer, designed and secured in place according to the buccal defect following a full thickness flap (Fig. 1H). Eleven weeks after surgery, the procedure seemed to yield acceptable clinical results since the postextraction horizontal bone defect appeared to be compensated. However, gingival redness was still present at the surgical site. At 11 weeks postgrafting, a gingival biopsy was collected at the grafted site for histological analysis, while uncovering the implant during the second stage surgery, as it involved the removal of the keratinized tissue (1–2 mm) covering the cover screw and the healing abutment placement.²¹ A prosthetic rehabilitation was performed 30 days later. Six months after the prosthesis placement, gum covering the grafted site was marked by a persistent redness that could be considered aesthetically unsatisfactory (Fig. 1I).

Histological Observations of Gingival Biopsies from the Second and Third Case

Biopsies collected during the surgical procedures (27 and 11 weeks after surgery, respectively) were routinely prepared for histological analysis on paraffin 5 µm sections.^{22,23}

Patient 2

Hematoxylin-eosin staining showed numerous elongated fibroblastic cells and round-shaped inflammatory cells invading fibrous tissue in the grafted site (Figs 2A and B magnifications ×26 and ×52, respectively). Verhoeff-Van Gieson staining revealed gingival epithelium, connective tissue, and alveolar mucosa (Fig. 2C, ×26). In the grafted area next to the alveolar bone, the deep connective tissue showed a red stained fibrillar material, indicating a very dense extracellular matrix. Higher magnification highlighted collagen and cell abundance consistent with a fibrotic process, as well as some shortened elastic fibers (Fig. 2D, arrows, ×52).

Patient 3

The histological examination of Hematoxylin-eosin-stained gingival sections revealed a well-defined border between pADM and the inflammatory gingival connective tissue (Fig. 3A, ×26). The blood vessels originating from both the flap and periosteum only invade the peripheral portion of pADM, indicating marginal neo-angiogenesis. At higher magnification, Verhoeff-Van Gieson staining highlighted the polymorphous inflammatory infiltrate including macrophages, lymphocytes, plasma cells, and foreign giant cell granulomas around lamellar structures (Figs 3B and C, ×52). Additionally, numerous fibroblast-like elongated cells were present in the ulceration, as well as newly formed collagen fibers. Periodic acid-Schiff red-stained lamellar structures (Figs 3D, ×26 and 3E, ×52) surrounded by foreign giant cell granulomas (Fig. 3F, ×52) could correspond to the pADM residues.

Ultrastructural and Histological Characterizations of pADM Prior to Implantation or Cell Culture

According to the manufacturer, the pADM used in the present study (Mucoderm® Botiss Biomaterials, Berlin, Germany) undergo a process that allows the decellularization and elimination of any potentially antigenic determinant, making them safe to use for humans. Scanning electron microscopy showed that the pADM surface displayed a very dense network including shallow pores, which corresponded to dermis depressions resulting from the loss of epidermal appendages during the preparative process (Figs 4A, ×50 and 4B, ×100). At higher magnification, pADM appeared to be composed of several dense layers of interconnected collagen fiber aggregates (Figs 4C, ×600 and 4D, ×1,000); some of them presenting characteristic periodic striations (Fig. 4D). Under polarized light microscopy and after picrosirius red staining, pADM displayed large collagen bundles (diameter >50 µm), with a few interfibrillar spaces (Fig. 4E, ×52) as compared to normal human gingiva.²⁴ At high magnification, the pADM collagen network seemed to be constituted of aggregated fibers forming large and abnormal wavy bundles (Fig. 4F, ×126).

Morphology and Distribution of Cultured Gingival Fibroblasts on pADM

Human gingival fibroblasts were obtained from gingival biopsies taken from six patients (18–30 years old) free of pathologies after obtaining their written informed consent in accordance with the principles outlined in the Declaration of Helsinki. Gingival fibroblasts were then cultivated on a piece of pADM in standard conditions. 10, 15, and 25 days after cell seeding, cultured pADM were prepared for standard histological procedure and specifically stained. The Hematoxylin-eosin staining showed an early onset of adherent fibroblasts on the pADM surface (Figs 5A to D). During

Figs 2A to D: Patient 2 grafted gingival site. (A) Inflammatory infiltrate; (B) Round inflammatory cells and other more elongated fibroblastic cells; (C) Very dense collagen network with some shortened elastic fibers; (D) Fibrotic area. (A, B) Hematoxylin eosin staining. (C, D) Verhoeff-Van Gieson staining. Ep. Epithelium, GCT. gingival connective tissue, mcl. muscle tissue. Scale bars: (A, C) = 100 µm, (B, D) = 50 µm

Figs 3A to F: Patient 3 grafted gingival site. (A) Connective tissue surrounding ulceration; (B) Ulceration showing foreign giant cell granulomas and lamellar structure attributed to pADM; (C) Giant foreign body type cells (arrows); (D, E) Ulceration containing the rest of pADM (red lamellar structures, arrows); (F) Giant foreign type cells (arrow); (A) Hematoxylin eosin staining; (B, C) Verhoeff-Van Gieson staining; (D to F) Periodic acid-Schiff staining. Scale bars: (A, D) = 100 µm, (B, C, E, F) = 25 µm

culture times, no deep pADM colonization by any of the used gingival fibroblast strains ($n = 6$) was observed except when cells

migrated in the collagen matrix fracture areas while remaining at the surface (Figs 5A and B, $\times 26$).

Figs 4A to F: pADM collagen network: scanning electron micrograph and picrosirius red staining. (A) Global ultrastructure of the pADM surface; (B) Surface depression in pADM: remains of skin appendage; (C) pADM dense collagen network; (D) pADM collagen bundles with periodic banding pattern; (E, F) Picrosirius red staining under polarized light. Scale bars: (E) = 200 μ m, (F) = 100 μ m

Ten days after seeding, the adherent cells were found unevenly distributed on the pADM surfaces and displayed a polygonal or elongated shape with round nuclei. At 15 days, some isolated pADM areas were occupied by confluent elongated fibroblasts (Fig. 5C, $\times 26$). After 25 days, fibroblasts formed a continuous layer over the pADM surface. Furthermore, some sites were occupied by fibroblast multilayers suggesting a preferential peripheral cell tridimensional layout and intercellular matrix synthesis (Fig. 5D, $\times 52$).

Collagen matrix remodeling of cultured pADM was evidenced by picrosirius red staining. Collagen material is stained in red under transmitted light and the fibrillar nature of the collagen network is revealed by its birefringence under polarized microscopy. In long-term cultures, the pADM portion in contact with cells is substantially degraded, as highlighted by picrosirius red staining (Figs 6A $\times 26$ and 6B, $\times 52$). At high magnification, the collagen hydrolysis was clearly observed at the pADM surface, while its inner part remained preserved (Fig. 6C, $\times 104$). Thus, under polarized light, the loss of birefringence revealed transformation of fibrillar collagen into gelatin, which demonstrated that the pADM peripheral area underwent time-dependent collagen hydrolysis over the course of the culture (Figs 6D to 6F, $\times 26$).

DISCUSSION

This study focuses on pADM bio integration as a gingival substitute in periodontal surgery mainly used for soft tissue augmentation.¹⁰ In fact, several studies reported the effectiveness of pADM and other soft tissue substitutes in improving the periodontal condition, emphasizing the favorable clinical aspect of volume augmentation,^{5–8} however, these procedures

require a better knowledge of wound healing, so that soft tissue substitutes are regarded as a completely viable option for gingival augmentation.^{25,26} Therefore, it seems necessary to understand the intimate mechanism of pADM biointegration in the rare situations associated with unfavorable gingival healing.²⁷ Thus, ideally, after *in vivo* grafting, pADM should be finally replaced by a newly formed gingival connective tissue.⁷ In order to do so, the exogenous matrix must be absorbed and invaded by cells (gingival fibroblasts, endothelial cells, and inflammatory cells) and blood vessels that allow tissue reformation.²⁸ Understanding the mechanisms leading to the biointegration of the exogenous matrix was crucial to fully interpret the poor clinical results observed in the current case reports. Thus, several months after the grafting procedure of both the first and third patients presented in this study were characterized by a persistent gingival redness and a partial loss of the reconstructed gingival volume. This is in accordance with a previous study showing a slower and incomplete healing when using pADM compared to autogenous CTGs.²⁹

This lasting connective tissue redness could be associated with chronic inflammation even though soft tissue substitute procedures are reported to incur a lower likelihood for postoperative pain or bleeding than autogenous procedures.^{8,29} Additionally, deep palpation revealed the presence of a denser than normal tissue that could be either nonresorbed pADM or newly formed fibrous tissue.

Thus, the histological examination of the third patient biopsy taken 11 weeks after surgery showed a persistent granulation tissue and inflammatory infiltrate within the grafted site. The chronic inflammatory reaction was marked by the presence of giant foreign body cells surrounding pADM as already reported with the same membrane after subcutaneous grafting in mice.³⁰ Curiously, even

Figs 5A to D: Cultures of human normal gingival fibroblasts on pADM. (A, B) Gingival fibroblasts on the pADM surface after 10 days; (C) Gingival fibroblasts on the pADM surface after 15 days; (D) Gingival fibroblasts form multilayers on the pADM surface after 25 days. (A to D) Hematoxylin eosin staining. Scale bars: (A to C) = 50 μ m; (D) = 25 μ m

though the second patient yielded acceptable clinical results, histological sections showed that 27 weeks after grafting, a clear fibrosis and a considerable inflammatory infiltration were associated with the pADM.³¹

Besides, this fibrous tissue was poorly vascularized and contained thick collagen fibers isolating the implanted xenogenic substitute from surrounding connective tissue, and that could be regarded as a later stage than it had been observed for the third patient. Aggregation and fusion of macrophages leading to the formation of a multinucleated foreign body reaction and associated with substantial fibrosis are one of the hallmarks of the foreign body response. It was obviously regarded as incompatible with successful tissue-engineering outcomes.³² This type of reaction usually occurs when foreign bodies cannot be eliminated by phagocytosis, enzymatic or inflammatory oxidative processes and need to be isolated.³³ Interestingly, lamellar structures highlighted by Periodic acid-Schiff reaction could show that some glycosylated materials remained in grafted pADM and explain, in part, the observed immune and inflammatory reactions. The bad biointegration could also be due to the inability of the tissue reaction to both achieve the pADM resorption and to allow both gingival fibroblasts and newly formed blood vessel invasions. As

previously shown adhesion, spreading, and proliferation of human gingival fibroblasts on the pADM surface demonstrated that specific and various collagen adhesion sites were still preserved and accessible for cell surface integrins.³⁴ However, the present study showed that, after 25 days of culture, the human gingival fibroblasts were still unable to invade the inner part of the pADM. In fact, the histological and ultrastructural studies performed before culture showed that the pADM collagen architecture differed from the normal gingival collagen network. Indeed, the histological appearance of normal gingival and dermal connective tissues, after picrosirius red staining,³⁵ was characterized by a 10–20 μ m range of fibrillar collagen fiber diameters with numerous and uniformly distributed interfibrillar spaces.³⁶ The abnormal appearance of pADM was characterized by very large collagen fibers (diameter >50 μ m) and a marked decrease of interfibrillar spaces. Furthermore, under scanning electron microscopy, the pADM displays a very thick and dense fibrous network without apparent interconnected porosity. Yet, other culture models showed that, after seeding, fibroblasts were able to deeply invade dense reconstructed collagen matrices without apparent pores. In these models, deep invasion and migration throughout the matrices were an MMP-dependent process involving cooperative activities

Figs 6A to F: Hydrolysis of cultured pADM collagen network by human normal gingival fibroblasts (picrosirius red staining). (A to C) Collagen degradation from the cultured surface (arrows) to the depth of pADM after 25 days (transmitted light); (D to F) Fibrillar collagen gradient of degradation characterized by birefringence loss (arrows) during culture course. (D) = 10 days, (E) = 15 days, (F) = 25 days (polarized light). Scale bars: (A, D, E, F) = 100 µm, (B) = 50 µm, (C) = 25 µm

of both collagenases (MMP-1) and gelatinases (MMP-2).³⁷ Under polarized light, picrosirius red staining showed a progressive loss of collagen birefringence from the surface to the internal part of the cultured pADM, evidencing transformation of fibrillar collagen network into gelatin. As expected, gradient degradation of the fibrillar collagen network may be due to collagenase secretion by proliferating gingival fibroblasts on the pADM surface. Thus, it can be clearly hypothesized that the resulting gelatin matrix was resistant to gelatinolytic activities, which prevents in-depth cell migration into the pADM membrane. Consequently, the proteolytic resistance of the membrane could be due to the formation of tight crosslinks between the collagen bundles during the manufacturing process and could explain the inability of surrounding tissue to integrate pADM. In addition, the highly dense pADM collagen network seems to limit *in vitro* cell colonization and both *in vitro* and *in vivo* tissue remodeling. Thus, inconsistent clinical results can be explained by a macromolecular pADM structure observably different from the physiological gingiva, despite their assumed common macromolecular composition.

CONCLUSION

The observed fibrotic process and the persistence of giant multinucleated foreign body cells evidence the poor ADM integration and the sustained inflammation of the grafted site. The usefulness of this kind of membrane is undeniable for soft tissue augmentation. However, its manufacturing process requires some improvements in order to achieve *in vivo* remodeling, and consequently better biointegration.

ETHICS

This research project "Poor biointegration of porcine ADM associated with unfavorable gingival healing: a report of three

cases," has been validated by the Ethics Committee of Saint Joseph University (ref: USJ-2013-17) in accordance with the Declaration of Helsinki.

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