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# Histopathological Analysis of Decellularized Porcine Small Intestinal Submucosa after Treatment of Skin Ulcer

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**Background:** Decellularized porcine small intestinal submucosa (SIS), commercialized as an extracellular matrix rich in cell-inducing substrates and factors, has been clinically applied to treat intractable skin ulcers and has shown therapeutic effects. The SIS reportedly induces cell infiltration and integrates with the ulcer bed after 3–7 days of application. The attached SIS degenerates over time, and the remaining mass appears as slough, below which is granulation tissue that is essential for healing. This study aimed to determine whether the slough should be removed in clinical settings.

**Methods:** Five patients with intractable skin ulcers were included in this case series. Seven days after applying a two-layer fenestrated-type SIS to the ulcer, the removed slough was histopathologically examined.

**Results:** The collagen fibers of the SIS somewhat degenerated, and inflammatory cell infiltration was observed from the ulcer side to the surface side of the SIS. Neovascularization was similarly observed on the ulcer side. The degree of inflammatory cell infiltration decreased from the ulcer side to the surface side, whereas pus (ie, aggregates of neutrophils) was observed on the surface and ulcer edges. Additionally, the removed slough contained regenerative epithelium on the ulcer side of the remaining collagen fibers.

**Conclusions:** After treating intractable skin ulcers using SIS, we recommend removal of the upper surface and ulcer edge of the degenerated SIS or slough to prevent infection and preservation of the lower side of the degenerated SIS to maintain the granulation tissue and regenerative epithelium. *(Plast Reconstr Surg Glob Open 2021;9:e3967; doi: 10.1097/GOX.0000000000003967; Published online 20 December 2021.)*

## INTRODUCTION

Various treatment modalities have been established for the treatment of intractable skin ulcers. Conservative treatment modalities (including occlusion dressing therapy, negative pressure wound therapy, and the use of artificial dermis) and surgical treatment modalities (including the use of flaps and skin grafts) are presently available. For ischemic ulcers, conservative treatment is preferred to prevent complications. Wound dressing materials help keep the wound adequately wet and preserve the granulation tissue. Biological materials are especially useful because they serve as scaffolds for the cells required for wound repair and have been clinically applied.[1–3]

Decellularized porcine small intestinal submucosa (SIS) is an extracellular matrix that maintains a three-dimensional structure, similar to that in the body.[4,5] Therefore, cells (such as fibroblasts and keratinocytes) easily migrate, extend, or adhere to it.[6,7] The SIS contains abundant cell-inducing substrates (such as elastin and laminin), extracellular substrates (such as fibronectin and glycosaminoglycans), and growth factors such as fibroblast growth factor-2, transforming growth factor-β1, and vascular endothelial growth factor.[8–10] The SIS product document describes that the materials should not be detached from the ulcer because they are

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**Table 1. Patient Background**

| | Age | Gender | Disease | Site | Complications | EVT Times during Treatment |
|---|---|---|---|---|---|---|
| Case 1 | 80 | Man | Ulcer after hematoma | Leg | DM HT CRF (HD) | None |
| Case 2 | 79 | Man | CLI | Toe | DM HT | 1 |
| Case 3 | 70 | Man | CLI | Toe | DM | 1 |
| Case 4 | 72 | Man | CLI | Toe | DM HT | 3 |
| Case 5 | 82 | Man | Diabetic ulcer | Toe | DM | None |

CLI: Critical lower limb ischemia; CRF (HD): Chronic renal failure (Under introduction of dialysis); DM: Diabetes; HT: Hypertension.

**Takeaways**

**Question:** Should the slough (degenerative SIS) be removed or not?

**Findings:** We analyzed slough histopathologically and, unlike the current recommendation of layering SIS, we recommend that slough on the ulcer edge and the superficial layer should be removed to promote good healing.

**Meaning:** The slough on the ulcer edge and superficial layer should be removed.

integrated with the ulcer bed. Our preliminary observation suggests that the SIS attached to the ulcer bed degenerates within 3–7 days; however, it is not fully integrated with the wound. The attached SIS degenerates over time, and the remaining mass appears as slough. Although several studies have histopathologically analyzed wound beds treated with the SIS,[1] in vivo analysis of the SIS itself has never been conducted after its application. Therefore, this study aimed to histopathologically analyze cells infiltrating the SIS and determine whether the slough should be removed.

## METHODS

### Patients

Five patients treated for refractory ulcers using the SIS were included in this study. These included three cases of critical lower limb ischemia (toe), one case of a post-hematoma ulcer (leg), and one case of a diabetic ulcer (toe) (Table 1). Endovascular treatment (EVT) was performed in three patients; this included percutaneous old balloon angioplasty in two patients and percutaneous old balloon angioplasty with stent grafting in one patient. Written informed consent was obtained in advance from all patients, and the research protocol was approved by the institutional ethics committee of Ota Memorial Hospital, Japan (No. OR19019).

### Treatment

The wound was constantly checked for blood flow. Percutaneous transluminal angioplasty was performed when hemodynamic parameters, such as the ankle brachial pressure index and skin perfusion pressure,

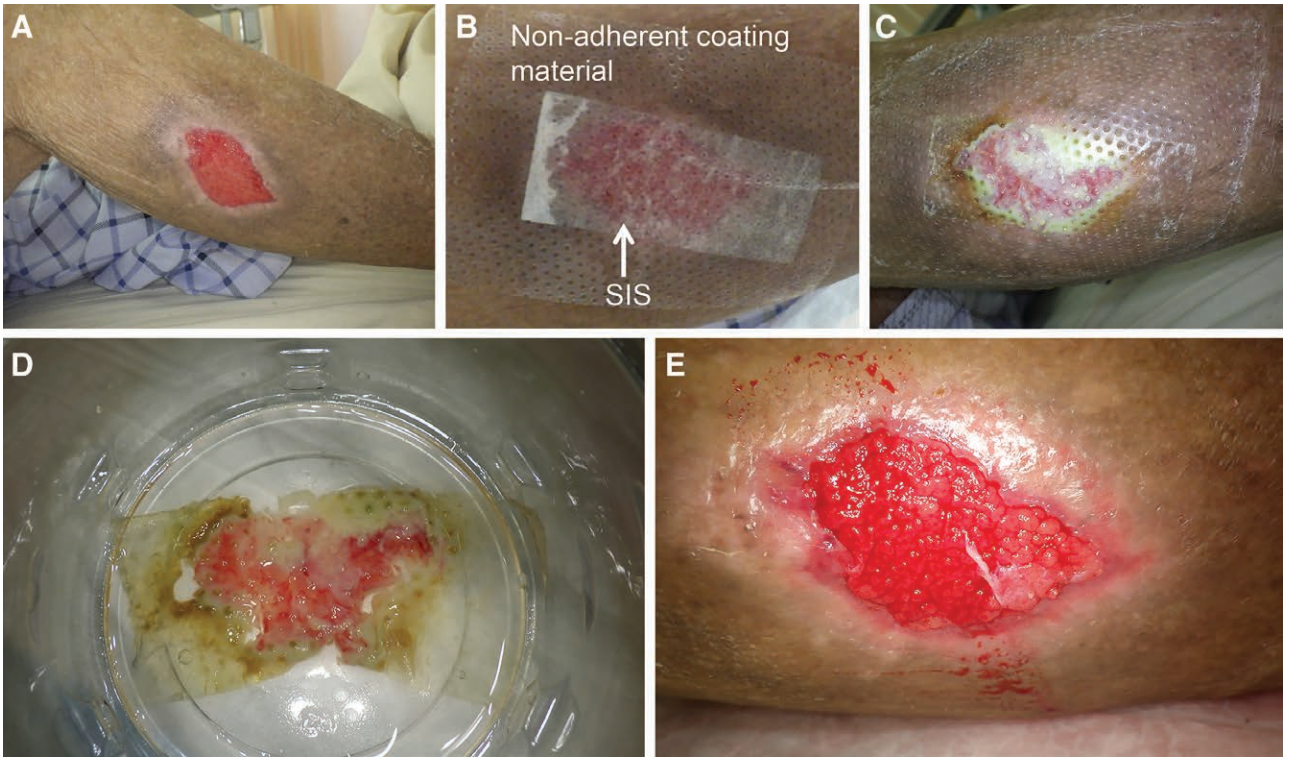


**Fig. 1.** From SIS attachment to slough retrieval in Case 1. A, Skin ulcer before treatment. Good granulation can be observed on the ulcer bed. B, SIS attachment. SIS was applied to the ulcer, and nonadherent coating material was layered on the SIS. C, Status 1 week after SIS application. SIS degraded and red granulation was visible at the center. The area of SIS that retained its structure was not in contact with the ulcer bed. D, Harvested slough 7 days after SIS application. E, Ulcer after slough removal, showing decreased size due to epithelium extending from the ulcer edge.

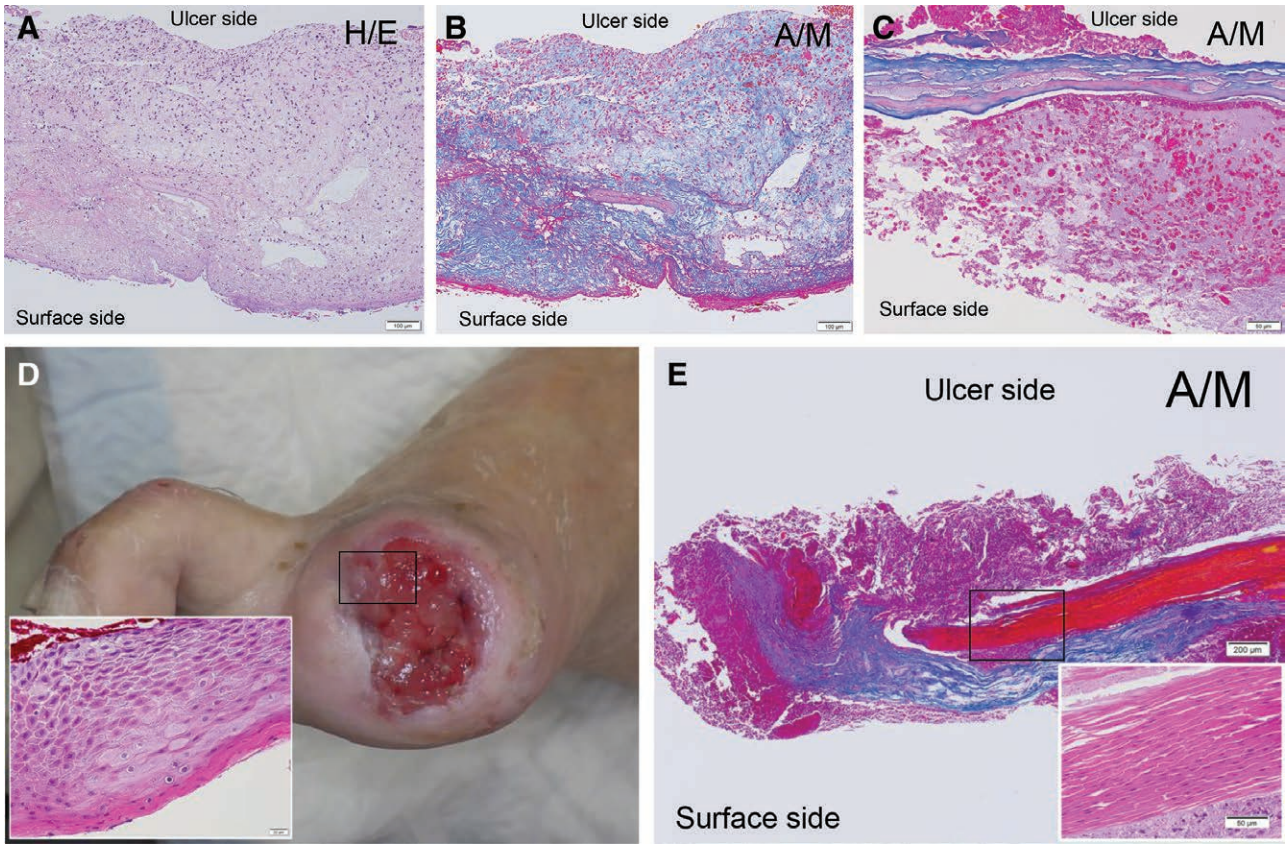


**Fig. 2.** Histopathologic analyses of slough and regenerative epithelium. A, Slough of Case 5. Inflammatory cells diffusely infiltrated into the slough and remained on its surface side. B, Slough of Case 5. The slough consisted of degenerative collagen fibers with diffuse inflammatory cell infiltration. Collagen fibers are stained blue by A/M stain. C, Slough of Case 3. A part of SIS-derived collagen fibers scarcely degraded, in which little cellular infiltration was observed. Inflammatory cells passed through SIS and accumulated on the surface. Collagen fibers are stained blue by A/M stain. D, Epithelialization of skin ulcer in Case 4. Slough was removed from ulcer 7 days after SIS application. Epithelium extension was observed from the ulcer edge. Complete structure of epithelium, from the basal layer to the stratum corneum, was observed histopathologically (inset: H/E). E, Slough of Case 4. Regenerative epithelium was observed on the ulcer side of mildly degraded SIS-derived collagen fibers. Squamous epithelium is stained red and collagen fibers are stained blue by A/M stain. The epithelium lacked a basal layer, had faintly-stained nuclei, and revealed parakeratosis (inset: H/E).

indicated reduced blood flow in the wound. Before SIS placement, the necrotic tissue was completely removed from the wound. Gangrenous tissue, such as gangrenous toe, was surgically resected. Suspected infection was treated with antibiotics and ointments, including sulfadiazine silver (GEBEN cream 1%, Mitsubishi Tanabe Pharma Corporation, Japan) and povidone-iodine preparations (POVIDONE-IODINE GEL 10% MEIJI, Meiji, Japan). Wound cultures were regularly performed, and antibiotics—including cefazolin sodium (Cefazolin Sodium, Otsuka, Japan); ampicillin sodium (Viccillin, Meiji, Japan); sulbactam/ampicillin (Sulbacillin, Meiji, Japan); tazobactam, piperacillin hydrate (Tazopipe, Meiji, Japan); and meropenem hydrate (Meropenem MEIJI, Meiji, Japan)—were administered according to the results of sensitivity tests. The wounds were covered with effective granulation tissue before SIS attachment. As a product material of the SIS, OASIS extracellular matrix (two-layer fenestrated type; Cook biotech Inc., IN, USA) was attached to the ulcer, and Mepitel One (Mölnlycke Health Care, Sweden), which is a nonadherent coating material, was used for fixation. The procedures were as follows. First, the ulcer surface was cleansed by mechanical and chemical debridement (Fig. 1A). Second, the SIS was attached such that it protruded 3–5 mm from the ulcer edge. Third, the SIS was soaked in saline solution. Fourth, following coverage with a nonadherent coating material (Fig. 1B), the wound was covered with gauze and fixed with tape or bandage. Fifth, the wound was washed with saline over the coating material daily. Sixth, the degenerated SIS was carefully removed from the wound 7 days later (Fig. 1C, D, E).

### Histopathological Analysis

The obtained slough was quickly fixed in 10% neutral buffered formalin solution, embedded in paraffin, and cut into 4-μm-thick sections perpendicular to the surface. Hematoxylin/eosin (H/E) staining and Azan–Mallory (A/M) staining were performed. Immunostaining was performed to identify the following cell types: CD33 (clone PWS44, 1×, Leica) for neutrophils, CD68 (clone KP1, 1×,

DAKO) for macrophages, CD31 (clone JC70A, 1×, DAKO) for endothelial cells, vimentin (clone V9, 1×, Ventana) for mesenchymal cells, and α-smooth muscle actin (αSMA; clone 1A4, 1×, DAKO) for smooth muscle cells.

## RESULTS

Due to the collagen components of the SIS, it is stained pink by H/E staining and blue by A/M staining, and it is non-nucleated. The obtained slough is mainly composed of edematous and fragmented SIS-derived collagen fibers and inflammatory cells infiltrated from the ulcer side to the surface side (Fig. 2A, B). We found inflammatory cells accumulated on the surface of the SIS. The degree of collagen fiber degradation varied depending on the site: the area in contact with the ulcer tended to be well-degraded (Fig. 2A, B), whereas the area in contact with healthy skin barely showed degradation (Fig. 2C). The epithelium was observed to regenerate on the ulcer side of the collagen fibers in two cases (Cases 2 and 4); the regenerative epithelium was extended from the surrounding epidermis. Complete epithelialization was noted in part of the ulcer edge (Fig. 2D), whereas a part of the epithelium lacked a basal layer, had faintly stained nuclei, and comprised a stratum corneum showing parakeratosis (Fig. 2E). The cellular infiltrates were mainly neutrophils (positive for vimentin and CD33), macrophages (positive for vimentin and CD68), and fibroblasts (positive for vimentin) (Table 2). These cells diffusely invaded the degraded collagen fibers (Fig. 3A, B), with minimal invasion of fibers with lesser degradation (Fig. 2C). Macrophages tended to migrate on the ulcer side of the SIS in four cases (Fig. 3C), and neutrophils tended to accumulate on the surface of the SIS and the ulcer edge in all cases, albeit with a nonuniform distribution (Fig. 3D). Neovascularization of capillaries (positive for vimentin and CD31, negative for αSMA) or arterioles/venules (positive for vimentin, CD31, and αSMA) was found close to the ulcer surface in three cases (Cases 1, 2, and 5) (Fig. 3E, F).

## DISCUSSION

The SIS is lyophilized and decellularized porcine small intestinal submucosa. It retains the three-dimensional structure of collagen in vivo and is rich in cell-inducing substrates and factors, including proteins such as elastin and laminin; extracellular substrates such as fibronectin and glycosaminoglycans; and growth factors such as fibroblast growth factor-2, transforming growth factor-β1, and vascular endothelial growth factor. The collagen structure is porous and open and is deemed to function as a scaffold for infiltrating cells and matrices from the ulcer bed. In the present study, we observed that the exudate with inflammatory cells from the ulcer surface passed through the SIS, with residual inflammatory cells on the surface side in all cases. Considering the structure of the SIS, we expected that cells would invade intact collagen. Although many inflammatory cells infiltrated the degraded collagen fibers (Fig. 2A, B), few infiltrated the intact collagen fibers (Fig. 2C). This suggests that the exudate passed through the fenestrated structure of the SIS in the area where the SIS was not degraded rather than the pores of the collagen fibers. We speculate that the passage of exudate through the pores may cause collagen fiber degradation, which may lead to cellular infiltration. Neutrophils were mostly distributed in areas that were not in direct contact with the ulcer, such as the ulcer edge, and they were also abundantly distributed on the surface of the SIS (Fig. 3D). The cells that were pooled on the surface of the SIS mainly comprised neutrophils, which often aggregated at the ulcer edge in the form of pus (Fig. 3D). In contrast, macrophages were more abundant on the ulcer side, suggesting that granulation was formed from the ulcer surface (Fig. 3C). The structure of the SIS is reportedly suitable as a scaffold for vascular endothelial cell adhesion.[11] In this study, cell populations positive for CD31 (endothelial cells) were observed close to the ulcer surface in three cases (Fig. 3E, F). They contained luminal structures, some of which were surrounded by smooth muscle cells. Although the vessels originally included in the SIS also consisted of smooth muscle cells, they could be differentiated from newborn vessels based on the absence of nuclei. Therefore, neovascularization was confirmed. Two of the three cases wherein neovascularization was observed were not complicated by marked arterial stenosis in the lower extremity that would necessitate EVT, and cure was achieved after only one EVT session in the remaining case. This clinical information suggests that the hemodynamic status of ulcers may affect neovascularization.[12]

Based on these observations, we considered the clinical application of the SIS. Slough is composed of SIS-derived collagen fibers, inflammatory cells, and fibrin. Several reports suggest that slough should be removed to prevent infection and facilitate wound healing.[13,14] However, regenerative epithelium was observed within this structure in two cases in the present study; this suggests that the overall slough should not be removed to retain regenerative

**Table 2. Degree of Cell Infiltration into SIS**

| | Degradation of Collagen Fiber | Whole-cell Infiltration | Fibroblast | Macrophage | Neutrophil | Vascular Endothelial Cell | Vascular Smooth Muscle Cell | Epithelial Extension |
|---|---|---|---|---|---|---|---|---|
| Case 1 | 2+ | 2+ | 2+ | 2+ | 2+ | 2+ | 2+ | 0 |
| Case 2 | 2+ | 2+ | 2+ | 2+ | 1+ | 1+ | 1+ | 2+ |
| Case 3 | 0 to 2+ | 0 to 2+ | 1+ | 0 to 1+ | 1+ to 2+ | 0 | 0 | 0 |
| Case 4 | 0 to 1+ | 0 to 2+ | 2+ | 1+ | 2+ | 0 | 0 | 2+ |
| Case 5 | 2+ | 2+ | 2+ | 2+ | 2+ | 2+ | 0 | 0 |

0: none; 1+: mild; 2+: moderate.

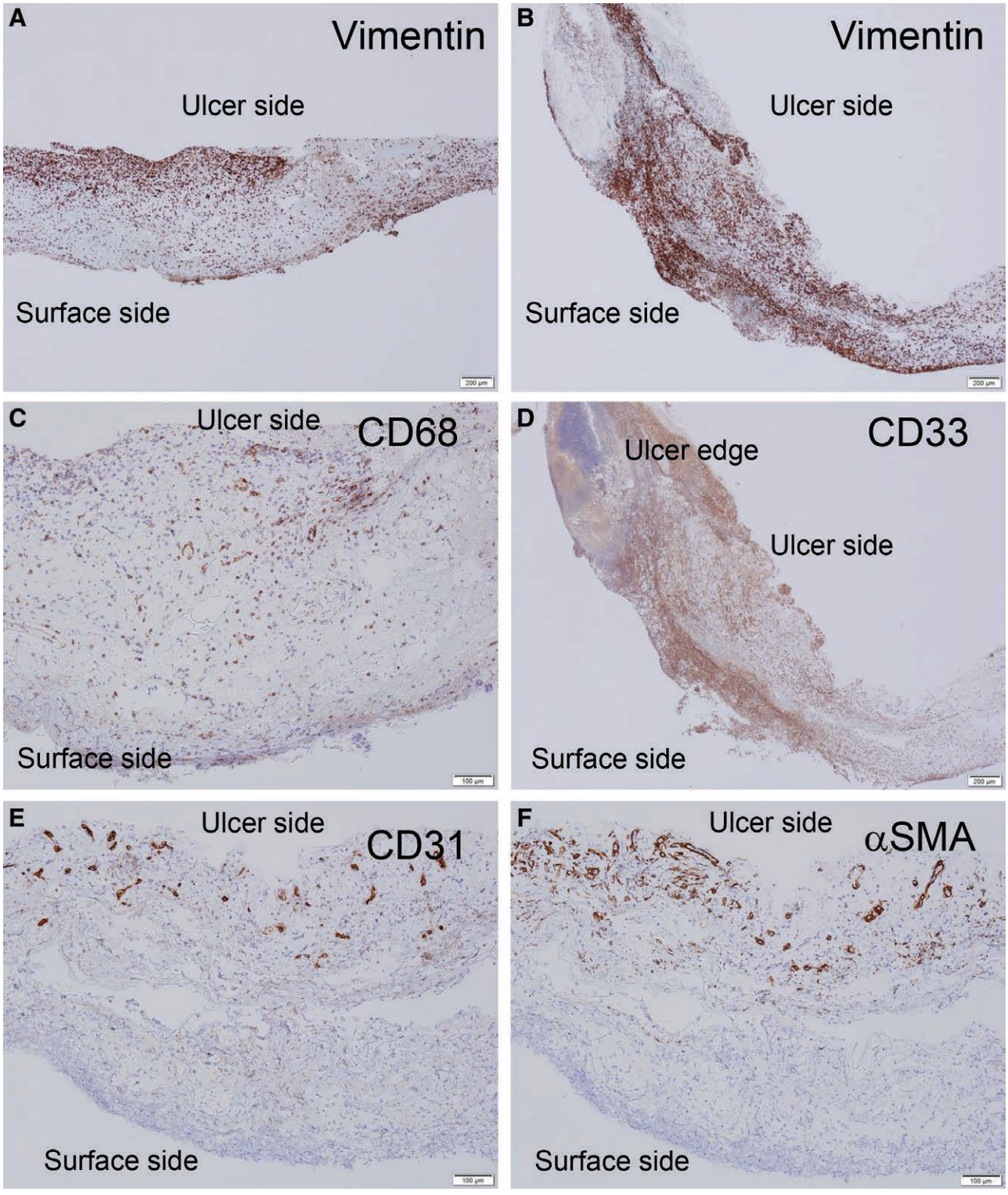


**Fig. 3.** Immunohistochemical analyses of cells infiltrating in the slough. A, Mesenchymal cells, which express vimentin, are densely distributed on the ulcer side and migrated towards the surface side (Case 5). B, Mesenchymal cells diffusely infiltrated in the degraded collagen fibers (Case 1). C, Diffuse macrophage infiltration was observed in the slough. (Case 5). Macrophages express CD68. Cell density decreased from the ulcer side to the surface side. D, Pus in the slough of Case 1. Pus was observed on the ulcer edge and surface side of the slough. Neutrophils express CD33. E, Neovascularization on the ulcer side of the SIS (Case 1). Vascular endothelial cells express CD31. F, Neovascularization on the ulcer side of the SIS (Case 1). The microvessels contain smooth muscle cells, suggesting that they are either arteriole or venule. Smooth muscle cells are positive for αSMA.

epithelial cells. Meanwhile, there were aggregates of neutrophils (ie, pus) on the surface of the SIS and at the ulcer edge in all cases. Therefore, we speculate that the currently recommended superimposition of the SIS may pose a risk of infection and delayed wound healing. Because macrophages and fibroblasts, which play key roles in wound healing, were abundantly distributed on the ulcer side of the SIS, and regenerative epithelium directly extended under the SIS, it may be beneficial to remove the portions that protrude from the ulcer edge and the surface layer. We recommend removal of the upper surface and ulcer edge of the degenerated SIS or slough to prevent infection and preservation of the lower side of the degenerated SIS to maintain the granulation tissue and regenerative epithelium.

This study was limited by the small sample size. However, it was a pilot study, and we observed that pus was formed at the ulcer edge and the SIS surface in all cases. Based on this result, we are planning a future study that will involve removal of the upper layer of a degenerated SIS for the promotion of wound healing in a large number of cases.

## CONCLUSIONS

In summary, histopathological analysis of the two-layer fenestrated-type SIS used for the treatment of intractable skin ulcers showed diffuse infiltration of inflammatory cells from the ulcer side of the SIS, whereas regenerative epithelium extended from the surrounding epidermis. Because pus formation was often observed on the SIS surface and at the ulcer edge, we recommend removal of those areas.

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