

Staphylococcus Aureus Infection Induces an Accumulation of Dibromotyrosine in the Thickened Superficial Layer of Chronic Cutaneous Wounds

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Abstract: Chronic wounds are often infected with *Staphylococcus aureus*. Such an infection causes deterioration of tissue repair mechanisms. Wounds are composed of granulation tissue and a superficial cover layer consisting of necrotic tissue and inflammatory cell infiltrates. In the present study, we examined 30 patients with skin wounds, with or without *Staphylococcus aureus* infection. The patients were divided into 3 groups according to the number of *Staphylococcus aureus* at 10⁵ CFU/g: lower value group (SA-I), and higher value group (SA-II). The thickness of the superficial layer and *Staphylococcus aureus* invasion were morphometrically analyzed. *Staphylococcus aureus* localized mostly in the superficial layer. The superficial layer becomes thicker in accordance with the increase in the number of *Staphylococcus aureus*. Patients in the SA-II group exhibit a significantly thicker superficial layer than those in SA-I group ($p = 0.0001$); however, no difference was found between the control and *Staphylococcus aureus*-I groups. Furthermore, the distance of *Staphylococcus aureus* invasion, measured histologically, was also significantly greater in SA-II than I ($p = 0.003$). In the superficial layer, myeloperoxidase (MPO)-expressing neutrophils and MPO producing dibromotyrosine (DiBrY), an oxidative product, were increasingly accumulated in accordance with the number of *Staphylococcus aureus* in the wound. These results suggest that DiBrY accumulation, which contributes to superficial layer thickening, is caused by the interaction between the infecting *Staphylococcus aureus* and the MPO-expressing activated neutrophils, which may form a biofilm in vivo. These events create poor conditions for tissue repair in chronic wounds.

Key words: *Staphylococcus Aureus*, Biofilm, Chronic Wound, Dibromotyrosine, Myeloperoxidase

Introduction

Staphylococcus aureus is one of the most common skin bacteria. It is a human bacterial pathogen associated with various wound conditions^{1,2,3} such as diabetic wounds, venous stasis ulcers, and pressure ulcers. Chronic wound infections are classified into 3 stages^{4,5}: i) wound contamination with organisms adhesion; ii) wound colonization without host injury by replicating microorganisms; and iii) wound infection with host injury by replicating microorganisms^{1,2}. The healing

process in infected wounds is also strongly influenced not only by the quality and quantity of microorganisms^{4,6,7}, but also by the patients' immunological capacity^{8,9}. However, the relationship between local immunity and infecting microorganisms is unclear. Biofilms are known to be produced by a number of bacteria, including *Staphylococcus aureus*¹⁰⁻¹⁴, and are ubiquitously formed even inside the body. Bacterial biofilms form an extracellular polymer matrix that supports the growth of bacterial communities¹⁵. In biofilms, bacteria gain a unique survival strategy—a form of cell-to-cell communication called “quorum sensing”^{16,17}. Biofilms also

act as a physical barrier against antimicrobials and attacks from host cells including neutrophils¹⁵. However, the characteristic of biofilm formed in the human wound is not known. In the present investigation, we picked up a total of 23 patients with chronic skin wounds and examined the relationship between human wound reaction and bacterial plexus, that's, biofilms on granulation tissues. Our results showed that *Staphylococcus aureus* infection increased the thickness of the superficial cover layer of the granulation tissue, in which neutrophil accumulation and tyrosine halogenation¹⁸ occur. These results might indicate the presence and the characteristic of biofilm through human cutaneous wound reaction.

Materials and Methods

Patients and ulcer tissue

Patients with skin wounds caused by burns, pressure ulcers, traumatic, postoperative surgical ulcers were admitted to the Fukuoka University Hospital. In this study, in spite of illness causes, for the sake of examining local wound tissue reactions for wound infection after onset, as a result, we selected cases including burns, pressure ulcers, surgical ulcers, which possessed various local immunity. Bacterial existence of the wound was ascertained by the swab culture method. At the same time, four samples (5-mm-punch biopsy, aseptic instrument) from a representative lesion within 1 cm² of the swabbing wound, were taken and two samples were used for histological examination, other two samples, frozen, later used for bacterial quantitative analysis in culture. Two blocks were used for histological examinations, each of them, formalin-fixed and frozen

with liquid nitrogen. We picked up 23 patients with only *Staphylococcus aureus* detected, as confirmed by swab culture. Bacterial detection was inspected by clinical bacterial examination department of Fukuoka University Hospital. Seven patients without bacterial detection were used as controls. The profiles of study patients are listed in Table 1. In the control and *Staphylococcus aureus*-detected groups, no significant differences in sex ratio, age at biopsy, or time from onset to biopsy, were found (Table 1). Patients with bacterial burden of *Staphylococcus aureus* were subdivided using 10⁵ colony-forming units (CFU)/g as a critical value of delayed wound healing, as documented previously⁴.

Institutional review board approval

This study was approved by the appropriate ethics committee of Fukuoka University Hospital. All patients provided written informed consent.

Bacterial quantitative analysis

Bacterial quantitative analysis was performed by using the serial 10-fold dilution method⁶. The biopsied tissues were weighed and homogenized, and the homogenates were serially diluted up to 10⁶ times. The diluted homogenate was spread onto gel plates of Mannitol Salt Ager (Japan BD Bioscience, Tokyo, Japan) and incubated at 37°C for 24 hours under aerobic conditions. After incubation, the bacterial colonies were counted and total bacterial numbers were expressed as CFU per gram of tissue.

Histological examinations

Formalin-fixed tissue specimens were embedded in

Table 1. Clinical profiles of patients with chronic skin wounds.

| | Control | SA I | SA II |
|--|------------------|------------------|----------------|
| Patients | 7 | 18 | 5 |
| Age (range) | 52.5y.o. (10-83) | 57.1y.o. (12-87) | 62y.o. (48-72) |
| Sex (M : F) | 4 : 3 | 16 : 7 | 3 : 2 |
| Duration from onset to biopsy (months) | 2.0 ± 1.9 | 3.3 ± 5.3 | 3.0 ± 1.3 |
| Cause of chronic ulcers | | | |
| Burn | 4 | 4 | 3 |
| Pressure ulcer | 1 | 8 | 1 |
| Post operative | 2 | 7 | 0 |
| Traumatic | 0 | 4 | 1 |

paraffin. Wound tissue was vertically cut into 12 thin serial cross-sections. The 1st, 4th, 7th, and 10th sections were used for hematoxylin-eosin (HE) staining; the 2nd, 5th, 8th, and 11th for gram staining; and the 3rd, 6th, 9th, and 12th for immunostaining using monoclonal antibodies against myeloperoxidase (MPO) (Dako Japan, Tokyo, Japan) and dibromotyrosine (DiBrY) (Nikken Ceil Co. Ltd., Shizuoka, Japan). In brief, paraffin-embedded tissue was cut to 4- μ m thickness. After deparaffinization, the sections were immersed in 1% bovine serum albumin (BSA) (Sigma-Aldrich Japan K.K., Tokyo, Japan) for 30 minutes, and then incubated at room temperature in a 1:100 dilution of MPO antibody and 10 μ g/mL DiBrY antibody for 1 hour. DAKO EnVision System (Dako Japan) was used for visualization. For fluorescence immunostaining, the second sample was embedded in optimal cutting temperature (O.C.T.) compound (Sakura Seiki K.K., Tokyo, Japan) and cut to 5- μ m thickness using a cryostat (CM 3050; Leica, Bensheim, Germany). After washing 3 times with 0.01 M, pH 7.2 phosphate buffered saline (PBS), the sections were immersed in 1% bovine serum albumin (BSA) for 30 minutes. The sections were then incubated at room temperature in a 1:50 dilution of mouse anti-*Staphylococcus aureus* IgM antibodies (Chemicon, Temecula, CA, USA) for 2 hours. After washing 3 times with PBS, the sections were incubated in rhodamine-labeled anti-mouse IgM (Rockland Immunochemicals, Inc., Gilbertsville, PA, USA) as the secondary antibody. To enhance the tissue structure by providing a clearer background, sections were stained with FITC-ConA (Vector Laboratories, Burlingame, CA, USA). Immunostained sections were observed under a confocal laser scanning microscope (LSM 510; Carl Zeiss, Göttingen, Germany).

Morphometric analysis

All tissue biopsies showed granulation tissue under a cover layer composed of eosin-positive coagulated exudative materials with inflammatory cells. Several morphological elements seen in 40 \times magnification photographs of the tissue with the cover layer in vertical cut were measured using a computer-assisted morphometric analyzer (VH analyzer; Keyence, Osaka, Japan). The thickness of the cover layer and DiBrY distribution area was determined by the following formula: cover layer = area \times 2 / (upper length + lower length). To measure the distance from the surface of the wound to the *Staphylococcus aureus* infiltration site in the tissue, immunostaining was done. The stained area was then observed under high magnification (400 \times or more); the observed round clusters confirmed *Staphylococcus aureus*-specific morphology. Low-magnification photographs (100 \times) were taken for measurement. The morphometric software VH analyzer was used to determine the deepest *Staphylococcus aureus* penetration in the 4 sections of ulcer tissue; this was designated the *Staphylococcus aureus* infiltration distance.

Statistical analysis

All data were expressed as mean \pm SEM. Differences between groups were examined for statistical significance using the Student's t-test and 1-way variance analysis (ANOVA). A *p* value of less than 0.05 was considered statistically significant.

Results

***Staphylococcus aureus* quantitative analysis**

In the control group, the sensitivity of our detection technique represented less than the number of

Table 2. The number of infecting *Staphylococcus aureus* (SA) and the thickness of the superficial cover layer in chronic skin wounds.

| | n | SA count (CFU/g) | Thickness of coagulated-exudative layer (μ m) |
|-------------------------------------|----|---|--|
| Control | 7 | No detected | 113 \pm 22.9 |
| SA-detected specimens | 23 | 2.4 \times 10 ⁵ \pm 1.6 \times 10 ⁵ | 522.7 \pm 220.1 |
| SA- I < 10 ⁵ CFU/g | 18 | 5.3 \times 10 ³ \pm 2.8 \times 10 ³ | 209.1 \pm 38.4 |
| SA- II \geq 10 ⁵ CFU/g | 5 | 1.2 \times 10 ⁵ \pm 7.7 \times 10 ⁵ | 1840 \pm 1003* |

**p*<0.05: SA- II vs. control.

Fig. 1 (Manabe et al.)

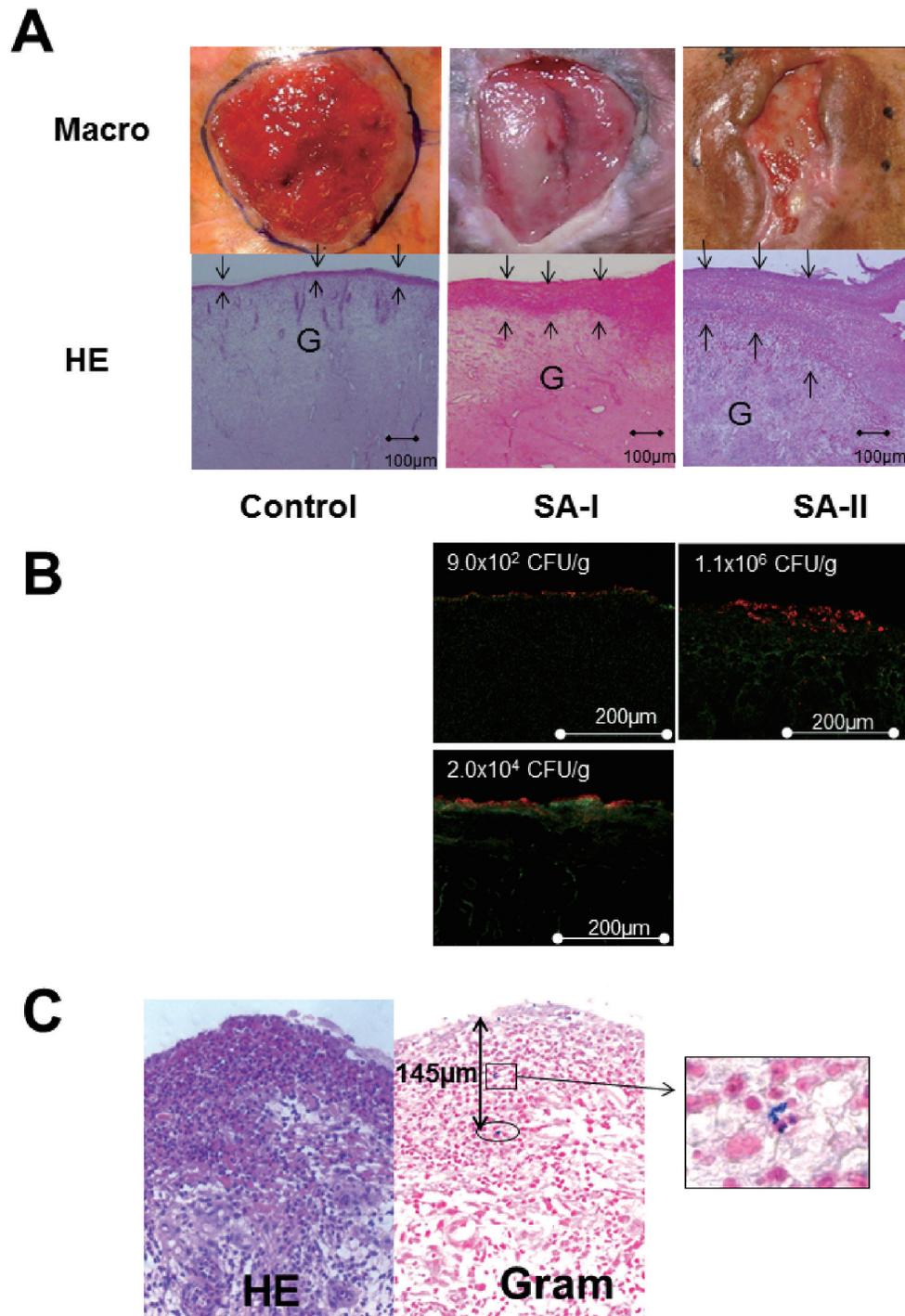


Figure 1: The superficial layer and localization of *Staphylococcus aureus* in chronic skin ulcers.

A: Macroscopic pictures and hematoxylin-eosin (HE)-stained sections of representative cases of chronic skin ulcers. Control group (a burn case with no bacterial infection), *Staphylococcus aureus* -I group (a pressure ulcer case), and II (a pressure ulcer case). Histologically, all lesions consist of a superficial cover layer (arrows) on the granulation tissue (G).

B: Colonization of *Staphylococcus aureus* in chronic wounds of representative *Staphylococcus aureus* -I and II cases. *Staphylococcus aureus* shown in red were superficially accumulated in chronic wounds.

C: Localization of *Staphylococcus aureus* detected by gram staining. *Staphylococcus aureus* shown by the magnified square were identified morphologically, and the deepest invasion distance was morphometrically measured as described in Materials and Methods. Original magnifications, $100 \times$ and $400 \times$.

Staphylococcus aureus in biopsy specimens (Table 2). In the *Staphylococcus aureus*-detected group (n = 23), the mean *Staphylococcus aureus* count was 2.4×10^5 CFU/g. *Staphylococcus aureus*-infected patients were divided into groups according to *Staphylococcus aureus* number at 10^5 CFU/g: SA-I group ($< 10^5$ CFU/g), mean *Staphylococcus aureus* count: 5.3×10^3 CFU/g; SA-II group ($\geq 10^5$ CFU/g): mean *Staphylococcus aureus* count, 1.2×10^6 CFU/g (Table 2).

***Staphylococcus aureus* infection and thickness of covered layer on granulation tissue**

Biopsy specimens from chronic wounds showed various degrees of repair responses such as the presence of granulation tissue, inflammatory cell infiltration, neovascularization. Both in control and *Staphylococcus aureus*-infected groups, granulation tissues were covered by an eosinophilic necrotic/coagulated layer with inflammatory cell infiltrates (Fig. 1A). The mean cross-sectional thickness of the cover layer in the control group was $113 \pm 22.9 \mu\text{m}$, which was 5 times thicker in the *Staphylococcus aureus*-infected group ($522.7 \pm 345.6 \mu\text{m}$) (Table 2).

Analysis of the relationship between the cover layer thickness and the *Staphylococcus aureus* count detected by the culture method revealed that the cover layer might become thicker as the *Staphylococcus aureus* count increased in the *Staphylococcus aureus*-infected group (Fig. 2A). SA-II patients had significantly thicker cover layers than SA-I patients ($p = 0.0001$) (Fig. 2B). However, no difference was found in the cover layer thickness between the control group and SA-I (Table 2 and Fig. 2B).

***Staphylococcus aureus* infection and invasion in chronic wounds**

After confirming gram positive bacteria by gramstaining, we histologically determined *Staphylococcus aureus* localization in wound tissues by immunostaining using a monoclonal antibody against *Staphylococcus aureus*. Although the immunofluorescence method should be quite sensitive, the detection power for bacterial visualization of the 2 methods was almost the same. In almost all specimens excluding 1 case, *Staphylococcus aureus* colonized within the cover layer (Fig. 1B). We measured the invasion distance of *Staphylococcus aureus* from the wound surface (Fig. 1C) and compared this distance with the thickness of the cover layer. A positive

Fig. 2 (Manabe et al.)

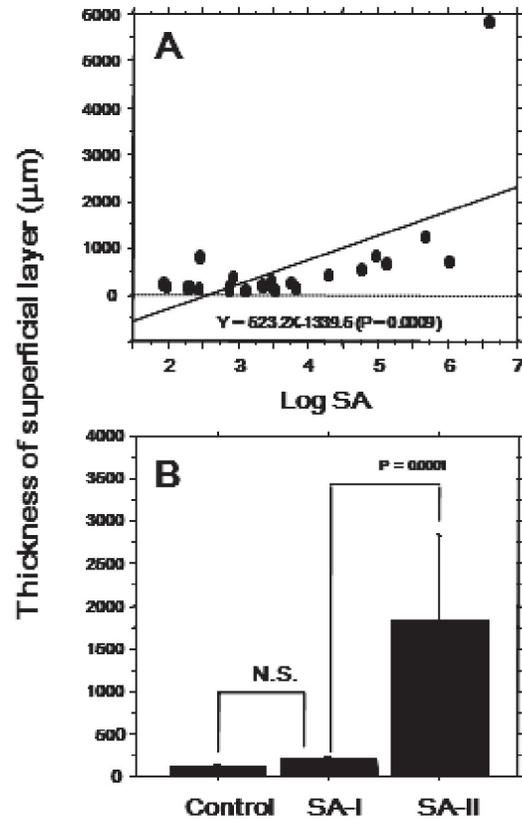


Figure 2: Relationship between the number of infecting *Staphylococcus aureus* and the thickness of the superficial layer.

A: Regression analysis between the 2 factors. B: The thickness of the superficial layer was compared among the control, *Staphylococcus aureus*-I, and II groups.

correlation between the 2 parameters was found (Fig. 3A). The distance of *Staphylococcus aureus* invasion was more than 7 times greater in SA-II than I ($347.4 \pm 325.1 \mu\text{m}$ and $48.2 \pm 43.3 \mu\text{m}$, respectively) (Fig. 3B).

Neutrophils and DiBrY accumulation in infected superficial layer of wounds

Local immunity may also be involved in the development of a thickened cover layer in *Staphylococcus aureus*-infected wounds. To detect neutrophils, MPO expression was analyzed. MPO-expressing neutrophils were intensively accumulated in the cover layer (Fig. 4A), and the extent of accumulation was correlated with the *Staphylococcus aureus* count (Fig. 4B). We then focused on an immunity-related oxidative product, DiBrY, which is

Fig. 3 (Manabe et al.)

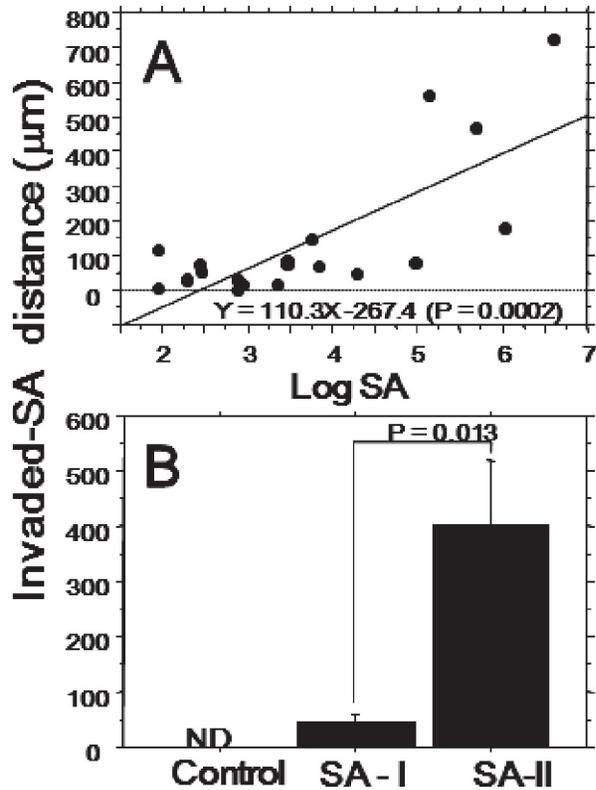


Figure 3: Relationship between the number of infecting *Staphylococcus aureus* and the deepest invasion distance in chronic ulcer tissue.

A: Regression analysis between the 2 factors.

B: The deepest invasion distance was compared among the control, *Staphylococcus aureus* -I, and II groups.

produced by peroxidases from neutrophils. When chronic wounds were examined by an immunohistochemical technique, DiBrY was found to be densely accumulated in the cover layer (Fig. 4A), and the extent of accumulation correlated well with the *Staphylococcus aureus* count in wounds (Fig. 4B). DiBrY accumulation was significantly correlated with increase in MPO-expressing neutrophils (Fig. 4B).

Discussion

In the present study, we showed association between increase in *Staphylococcus aureus* count in chronic cutaneous wounds, and thickening of the superficial cover layer of the granulation tissue. This layer is

formed by exudated plasma contents immediately after wound formation^{19,20} and act as a biological occlusive dressing that promotes wound healing by accelerating reepithelialization^{11,21}. The cover layer can also be used as a matrix for bacterial attachment and proliferation^{19,23}. We divided the patients into groups according to the number of infecting *Staphylococcus aureus* burden at 10⁵ CFU/g of ulcerative tissue. This value has long been controversial in acute and chronic situations^{9,22}. However, we adopted this value as a critical number to estimate the effects of *Staphylococcus aureus* burden in chronic wounds from our experience. Interestingly, *Staphylococcus aureus* was found mostly in the superficial layer, and the thickness of this layer was increased by the increase in the number of *Staphylococcus aureus*. Moreover, the invasion distance from the surface increased as the *Staphylococcus aureus* number increased. The thickening of the superficial layer possibly occurs as a result of immunological responses to infecting *Staphylococcus aureus*. Indeed, neutrophils were greatly accumulated in the superficial layer compared to the granulation tissue under the cover layer. Biofilms have emerged as communities of bacteria in many medical conditions, and are associated with chronic wounds^{3,10,13,23}. The exact character of biofilm in human tissue has not been defined because specific antibodies or tracers to examine certain biofilms are thus so far not available. However, through chronic human wound reactions, the existence of biofilm including various kinds of materials^{3,13,14}, extracellular polysaccharide, glycocalyx, microbes, toxin, might be shown. Therefore, the superficial cover layer of the granulation tissue is the demarcation zone between the bacteria and the host tissue. MPO localized in the phagosome of neutrophils produces hypochlorous acid, which acts as a strong bactericide. MPO-expressing neutrophils greatly accumulated in the *Staphylococcus aureus*-infected superficial layer. We focused on a peroxidase-related product, DiBrY, as an end product of oxidation¹⁸. The production of DiBrY, which was densely accumulated in the superficial layer, is catalyzed by MPO or eosinophil peroxidases (EPO). These results suggested that DiBrY, the accumulation of which thickened the superficial layer might be associated with the interaction between the infecting *Staphylococcus aureus* and the MPO-expressing activated neutrophils. Such complex structures may form biofilms in vivo and create poor conditions for tissue repair in chronic wounds. In conclusion, we examined human chronic cutaneous ulcers with *Staphylococcus*

Fig. 4 (Manabe et al.)

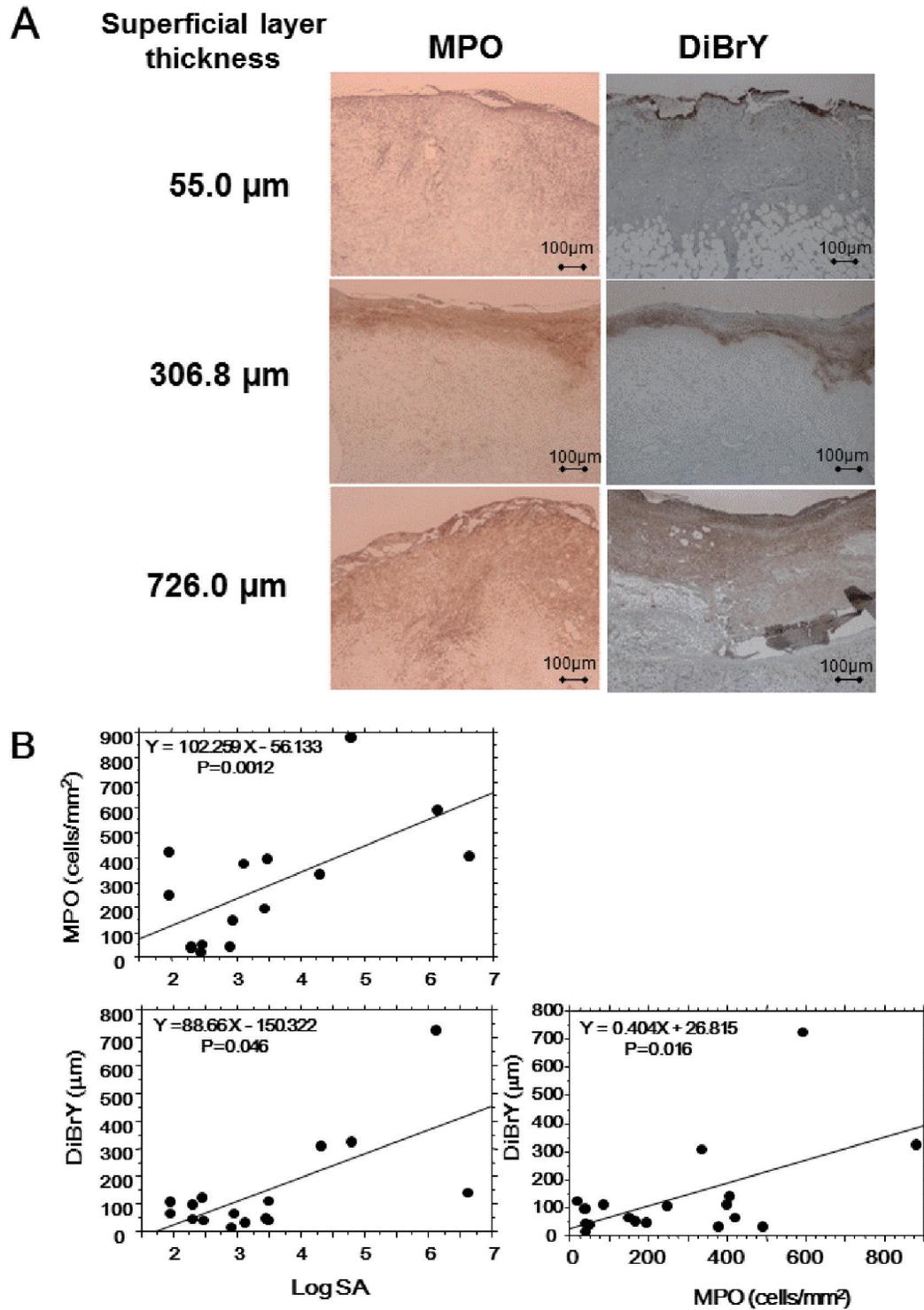


Figure 4: MPO-expressing neutrophils and DiBrY accumulation in the superficial layer.

A: MPO-expressing neutrophils and DiBrY were accumulated in the superficial cover layer of the granulation tissue. In accordance with increase in the thickness of the superficial layer, infiltration of MPO-expressing neutrophils and DiBrY accumulation also increased. Scale, 100 μm .

B: Left; Relationship among the number of infecting *Staphylococcus aureus*, density of MPO-expressing cell, extent of DiBrY accumulation. Right; Relationship between the density of MPO-expressing cell and extent of DiBrY accumulation.

aureus infection and found that the superficial cover layer was thickened by the accumulation of MPO-expressing neutrophils and DiBrY. Such devastating agent should be removed by debridement.

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