

Assessment of histopathology of wounds based on protein distribution detected by wound blotting

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Abstract

Background: Shortening the duration of healing based on an accurate assessment is important in pressure ulcer management. This study focused on the peroxidase and alkaline phosphatase activity detected by wound blotting, a non-invasive method of collecting wound exudate, to establish a non-invasive and point-of-care assessment method for analyzing the histopathology of wounds using an animal model.

Methods: Wounds were created on the dorsal skin of rats. Peroxidase and alkaline phosphatase activities in the wound exudate were detected by wound blotting on post-wounding days 1, 4, 7, and 10. Wound tissue was collected on the same sampling days. Peroxidase and alkaline phosphatase activity within the tissue and myeloperoxidase were visualized. Two types of peroxidase activities were detected by wound blotting: ring and non-ring signals. The histopathological features were compared between wounds with ring and non-ring signals.

Results: The wounds with ring signals showed a high level of peroxidase activity, and histological analysis demonstrated that the secreted or deviated peroxidase activity originated from myeloperoxidase, indicating a strong inflammation reaction within the tissue. The histopathology of wounds related to the alkaline phosphatase signals was not identified.

Conclusion: The results suggested that ring signals indicated a strong inflammatory reaction and that they could be used to assess non-visible inflammation.

Keywords

Assessment, histopathology, wound

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Introduction

A pressure ulcer (PU) is a critical health-care issue, as it can cause pain, produce unpleasant odors, lead to increased risk of septicemia, and decrease the quality of life. In addition, there is the burden of medical costs for PU treatment.^{1–4} In PU management, it is important to shorten the healing period by selecting an appropriate treatment and care based on an assessment of the PUs.⁵ Histological analysis using tissue biopsy specimens can help to understand the complex pathophysiology of PUs. PU management, however, requires repetitive assessment, and repetitive tissue biopsies are impossible due to the invasiveness of the procedure. Therefore, it is desirable to develop novel, non-invasive methods to assess the pathophysiology of PUs based on histopathological features.

Several studies have examined the relationship between proteins in wound exudate and wound healing.^{6,7} Various

types of cells regulate the wound-healing process via soluble factors such as cytokines, growth factors, and extracellular matrices (ECMs) in an autocrine and/or paracrine manner. Therefore, the wound exudate can possibly be substituted for the tissue lysate of a biopsy sample in the biochemical analysis used to examine the histopathology of wounds. We focused on wound blotting as a collection method of

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exudates.⁸ Wound blotting realizes collecting fresh exudate by attaching a piece of nitrocellulose membrane to a wound surface for 10 s. The protein signals detected by immunostaining of the nitrocellulose membrane can be consistent with the protein distribution within the tissue.⁹ We selected peroxidase and alkaline phosphatase (ALP) as biomarkers for a point-of-care assessment. Peroxidase and ALP are widely used for the enzyme labeling of antibodies, and several of their substrates are commercially available.^{10–12} The peroxidase and ALP activity in exudate can be visualized within a few minutes by reaction with chemiluminescence substrates; the activities can then be recorded by a portable imager. Therefore, peroxidase activity detection by wound blotting is a promising method for point-of-care assessment to examine the histopathology non-invasively, rapidly, and repetitively. We previously reported that the peroxidase activity was significantly related to the liquefaction of necrotic tissue of PUs.¹³ However, the histopathology of wounds analyzed by the peroxidase activity in exudate has yet to be examined. One candidate for the sources of peroxidase activity signal detected by wound blotting is myeloperoxidase (MPO). MPO is an important peroxidase related to neutrophils' phagocytosis¹⁴ and catalyzes the formation of hypochlorous acid which has a microbicidal activity when provided with H₂O₂. In addition, MPO is effused into the extracellular space as a component of neutrophil extracellular traps (NETs),^{15–17} where it retains its activity.¹⁸ Therefore, MPO can be a source of the peroxidase activity signal when it is released as a component of NETs or is deviated by the necrosis of neutrophils. Another candidate source of the peroxidase signal is glutathione peroxidase (GPx) 3, or plasma GPx,^{19,20} because GPx3 is released from circulation into the wound exudate.

ALP is an esterase that hydrolyzes a variety of monophosphate esters. The enzyme occurs widely in various tissues of the body.²¹ In the field of cutaneous wound healing, previous studies have reported an increased ALP activity in the regions of granulation tissue formation²² as well as in inflammatory cells in skin diseases with inflammatory infiltration associated with hyperplasia and fibroblastic reaction.²³ However, the role of ALP activity in cutaneous wound healing has not yet been fully described.

This pilot study aimed to reveal the sources of these enzyme activity signals and to clarify the histopathology of wounds related to the enzyme activity signals detected by wound blotting. First, we examined whether signal patterns or intensity detected by wound blotting indicated levels of enzyme activity using dot blot experiments. Then, we examined enzyme activity signals of cutaneous wounds in the wound-healing process. From results of this pilot study, we would be able to find what patterns of signals could be used as an indicator for assessment of wound by wound blotting. We used both young and aged rats in this study because we hypothesized that wounds with the same histopathological conditions demonstrated the same signal patterns of enzyme activity regardless of their physical status. Our goal is to

establish a non-invasive and point-of-care method of assessing the histopathology of wounds.

Materials and methods

Dot blot experiments

Peroxidase-conjugated immunoglobulin (Peroxidase-conjugated Anti-Rabbit IgG, JacksonImmuno Research, West Grove, PA) was diluted with distilled water at three different concentrations (80, 8, and 0.8 ng/mL) and then was dropped onto a piece of nitrocellulose membrane (Supported Nitrocellulose Membrane, 0.2 μ m, Bio-Rad, Hercules, CA; 3 and 10 μ L for each, respectively). Distilled water was used as a negative control. The pieces of membrane were dried for 10 min at room temperature, followed by soaking in 0.01 M phosphate-buffered saline (PBS). The membrane was reacted with peroxidase substrate solution (Luminata Forte, Merck Millipore, Darmstadt, Germany) in a chemiluminescence imager (LumiCube, Liponics, Tokyo, Japan). A chemiluminescent signal was recorded immediately and every 30 s after substrate application until 2 min with a 3-s exposure time.

ALP-conjugated immunoglobulin (Alkaline Phosphatase AffiniPure Donkey Anti-Goat IgG, JacksonImmuno Research, West Grove, PA) was also diluted with distilled water (60, 6, and 0.6 ng/mL) and dropped onto a piece of membrane in the same manner as that carried out for peroxidase. ALP substrate solution (Chemiluminescent AP Microwell/Membrane Substrate, SurModics, Eden Prairie, MN) was used to detect the ALP activity. Images of chemiluminescence were recorded with a 30-s exposure time every 1 min until 4 min from the application of the substrate solution.

Animals and wounding

A total of 20 9-week-old (young) and 20 6-month-old (aged) male Sprague-Dawley rats were purchased from Japan SLC (Shizuoka, Japan). We used both young and aged rats to analyze wounds under several physiological conditions. The rats were bred under the uniform conditions with a temperature of 23°C \pm 2°C, humidity of 45% \pm 10%, 12 h/12 h light/dark cycle, and ad libitum feeding. After a 1-week acclimatization period, the dorsal hair of the rats was shaved off. A full-thickness excisional wound measuring 2.5 cm in diameter was made on the dorsal skin, and then it was covered with polyurethane film and gauze. The wounds were washed by normal saline, and the dressing was changed once a day until either wound closure or tissue sampling. Also, researchers observed and evaluated appearance/disappearance of slough, contraction, and wound closure every day based on wound appearance (wound size, shape, and color of wound surface) using photographs of the wounds. All experimental procedures for the rats were conducted under the inhalation anesthesia (2% isoflurane for wounding and dressing change, 5% isoflurane for sampling, Pfizer, Tokyo, Japan.). Isoflurane has an analgesic effect.

The study protocol was approved by the Animal Experimentation Committee of the School of Medicine, the University of Tokyo. This study was conducted according to the Guidelines for Proper Conduct of Animal Experiment provided by the Science Council of Japan.

Detection of peroxidase and ALP activity by wound blotting

Wound exudate was collected on post-wounding days (PWDs) 1, 4, 7, and 10 using the wound blotting method described in a previous study with a slight modification.⁸ Briefly, a piece of nitrocellulose membrane was attached to the wound surface for 10 s. Before performing the wound blotting, we washed the wound and the surrounding skin by normal saline and wiped gently not to damage the wound surface. The membranes were stored at 4°C until analysis.

The membrane was immersed in 0.01 M PBS prior to peroxidase activity detection. The peroxidase activity was visualized by the same procedure used in dot blotting. After the peroxidase signal disappeared, the membrane was reacted with the ALP substrate solution for 4 min in the dark followed by image recording. All peroxidase and ALP signal images were flipped horizontally to line them up with the photographs of wounds using an image software program (GUN Image Manipulation Program, The GIMP Development Team, <http://www.gimp.org>). The images of ALP signals were divided into three channels of red, green, and blue (RGB), and blue channel images were used to measure the mean intensity of ALP signals on the whole wound area using the ImageJ software program (National Institutes of Health, Bethesda, MD).

Tissue and blood sampling

The wound tissue and blood samples were collected on PWDs 1, 4, 7, and 10 after wound blotting (n=5 for each sampling day). The tissue was divided into two pieces in the center of the wound. A head-side specimen was freshly frozen while the other side was fixed by 4% paraformaldehyde in phosphate buffer for paraffin embedding. Blood samples were collected from young rats. Blood serum and clots were separated by centrifugation (5000g × 5 min). Both the frozen tissue and serum samples were stored at -80°C until analysis. Blood samples were also collected from three 10-week-old rats without wounding.

Histological analysis

The paraffin-embedded tissue sections were stained with hematoxylin and eosin (HE) as well as Masson's trichrome stain (MT). The detailed histological characteristics, including level of inflammation and collagen synthesis, were described by an expert medical technologist using these staining sections. The technologist was blinded to the peroxidase and ALP activity signals.

To visualize the peroxidase activity in the tissue, the frozen sections were incubated with peroxidase substrate (VECTOR NovaRED Peroxidase Substrate Kit, Vector, Burlingame, CA) for 3 min. A pair of sections was prepared as a negative control, in which the peroxidase activity was inactivated by incubation with 0.9% H₂O₂ in methanol for 30 min before reaction with the substrate. The ALP activity in the frozen tissue was detected by incubation with ALP substrate (VECTOR Red Alkaline Phosphatase Substrate Kit, Vector) for 15 min. Negative control sections were incubated with the substrate solution supplemented with 4-mM levamisole, which is an ALP inhibitor. The frozen tissue sections were also used for immunohistochemistry of MPO and GPx3. The sections were fixed with 100% methanol, washed with PBS, and then incubated with 0.9 % H₂O₂ in methanol for 30 min at room temperature to inactivate endogenous peroxidase. After a 30-min incubation with blocking solution (1% bovine serum albumin in PBS), the sections were incubated with anti-MPO heavy chain antibody (1:50 dilution, sc-34159, Santa Cruz Biotechnology, Dallas, TX) overnight at 4°C and polymerized using secondary antibody labeled with horseradish peroxidase (Histofine Simple Stain Rat MAX PO (G), Nichirei Biosciences, Tokyo, Japan) for 30 min at room temperature. For the immunohistochemistry for GPx3, the sections were incubated with anti-GPx3 antibody (sc-50496, Santa Cruz Biotechnology) overnight at 4°C following the inactivation of endogenous peroxidase and then were reacted with peroxidase-conjugated anti-rabbit IgG (1:50 dilution, JacksonImmuno Research) for 30 min at room temperature. Then, 3,3'-diaminobenzidine (DAB) was used to visualize immunoreactivity. All sections for enzyme activity detection and immunostaining were counterstained with hematoxylin. All tissue section images were captured by an optical microscope (BZ-X710, Keyence, Osaka, Japan).

Peroxidase and ALP activity in serum

The peroxidase and ALP activities in serum were measured by the colorimetric method. A commercially available measurement kit (QuantiChrom peroxidase Assay Kit, BioAssay Systems, Hayward, CA) was used to measure the peroxidase activity according to the product manual. The ALP activity was measured using NBT/BCIP solution (1-Step NBT/BCIP, Thermo Fisher Scientific, Waltham, MA). The peroxidase activity level in serum were compared among rats with and without wounds using Kruskal-Wallis test. Pearson's correlation coefficient was used to measure the association between the ALP activity levels in serum and the intensity of ALP activity detected by wound blotting.

Clarifying histopathology related to the peroxidase and ALP activity detected by wound blotting

We categorized the histological description made by an expert medical technologist based on the features of cells and ECM, focusing on inflammatory signs and collagen synthesis. The

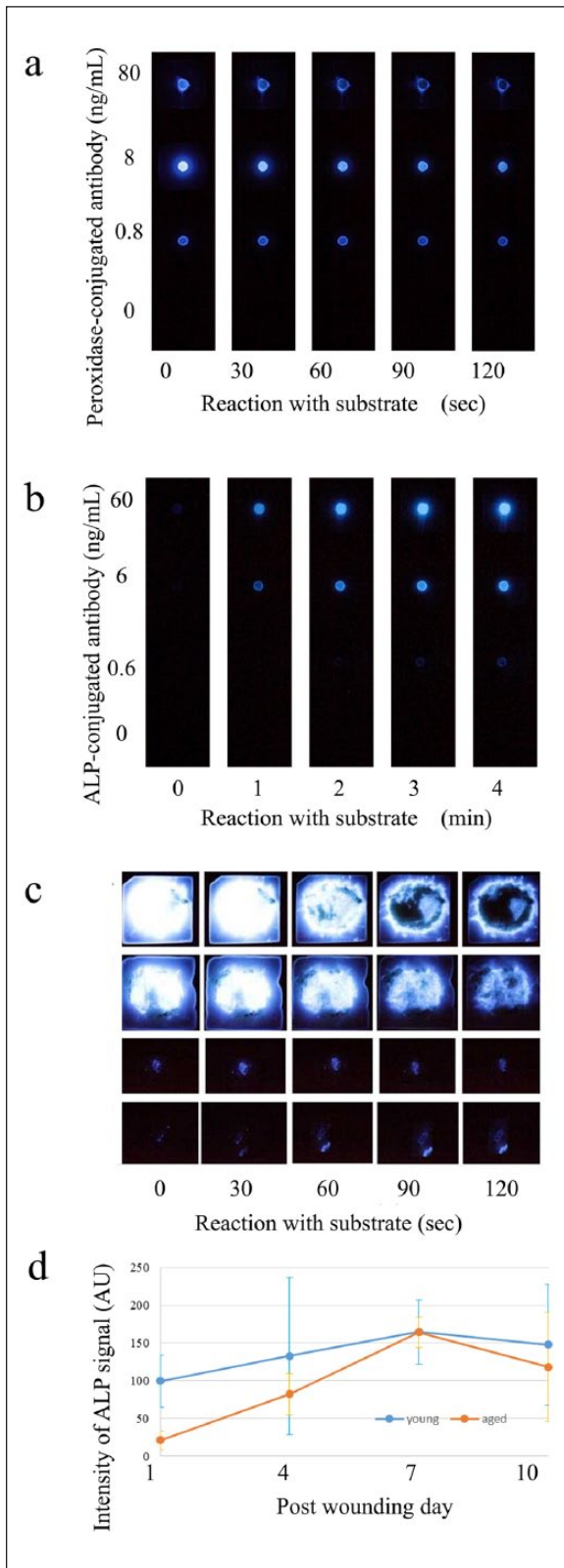


Figure 1. Peroxidase and alkaline phosphatase activity level and their signals. Peroxidase (a) and alkaline phosphatase (b) activity levels in the dot blot experiment are shown. (a) The signals of the highest concentration of peroxidase were weaker than those

Figure 1. (Continued)

of the second highest concentration. The signals weakened in a time-dependent manner. (b) The signals of alkaline phosphatase activity increased in a time-dependent and concentrate-dependent manner. (c) The peroxidase signals of various wounds detected by wound blotting are shown. The signals that were strong at 0s decreased in a time-dependent manner, and the signal at the wound edge (ring-shaped signal) remained constant. (d) The mean intensity of the alkaline phosphatase (ALP) activity signal detected by wound blotting increase from day 1 to day 7 in both young and aged rats. The data represent mean \pm SD; $n=5$ for each day.

wounds were grouped according to the peroxidase and ALP activity signals detected by wound blotting. For the analysis of the intensity of ALP signals, the upper one-third of the ALP intensity was used as threshold to divide the wounds into two groups. We compared numbers of wounds which showed each histological feature that was evaluated by an expert medical technologist between the two groups. Fisher's exact test was used to compare enzyme activity within tissues between the two groups.

Results

The peroxidase and ALP activity level and signals

Dot blot experiments were conducted to examine whether the signal intensities of the peroxidase and ALP activity were activity level dependent. The signal spread to the surrounding area and disappeared within the blotted area, producing the so-called "ring signal," in the 80 ng/mL peroxidase solution. Both 3 and 10 μ L dot blot experiments showed the same signal change (Figure 1(a)). The signal intensity of the ALP activity increased depending on the time of reaction and the concentration of ALP solution (Figure 1(b)).

Wound healing

The wounds on PWD 1 of young rats showed slough that was spontaneously debrided around day 4 (Figure 2). Wound contraction was observed from PWD 4 to 10. The wounds were completely closed by PWD 11 in all young rats. The disappearance of slough, contraction, and wound closure were delayed in the aged rats in comparison to the young rats. HE and MT staining showed abundant inflammatory cells on PWD 1 and 4 and fibroblast-like cells on PWD 7.

Peroxidase activity signals detected by wound blotting

The peroxidase activity detected by wound blotting showed a ring signal and a decreasing signal, similar to the findings obtained for dot blotting (Figure 1(c)). A ring signal was observed in all five wounds on PWD 1 in the young rats and

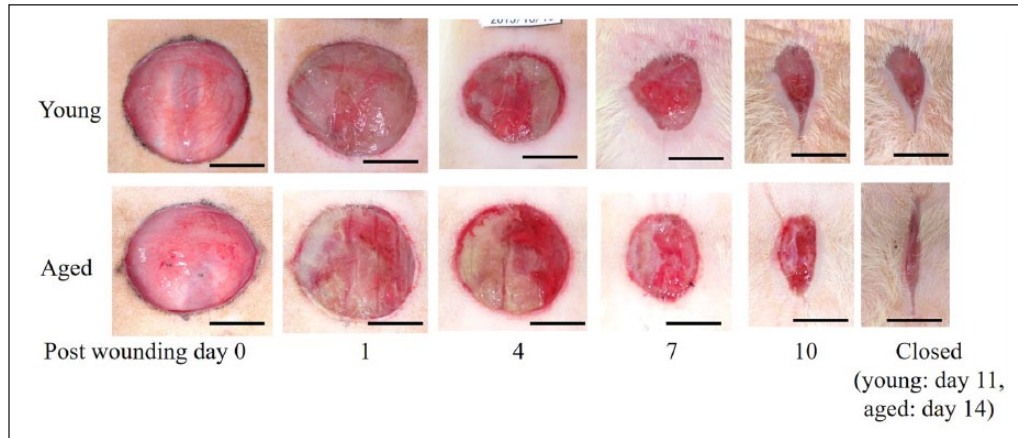


Figure 2. Macroscopic observation of cutaneous wounds in young and aged rats. The wounds of young rats showed slough disappearance earlier than in aged rats. Also, wound contraction was noticeable in young rats. The wounds closed by PWD 11 and 14 in young and aged rats, respectively. Bar: 1 cm.

Table 1. Peroxidase activity detected by wound blotting in the dorsal excisional wounds.

Post wounding day		Young				Aged			
		1	4	7	10	1	4	7	10
Peroxidase activity	Ring	5	1	0	0	4	4	0	0
	Non-ring	0	2	4	5	1	1	4	5

The excisional wounds were created on the dorsal skin of young and aged rats. Wound exudate was collected by wound blotting on post-wounding days (PWDs) 1, 4, 7, and 10, and the peroxidase activity was visualized using chemiluminescence. The signals of the peroxidase activity were classified into two types: a ring signal and a non-ring signal. Five rats were prepared for each PWD; however, four samples were excluded because of bleeding of the wounds.

in 4 of 5 wounds on PWD 1 and 4 in the aged rats (Table 1). Four samples were excluded from the peroxidase analysis of wound blotting because of the bleeding of wounds. There was no peroxidase signal through the 120-s reacting time in the bleeding area.

The serum samples collected from nine young rats were used for the analysis because the remaining samples had problems associated with either hemolysis or an insufficient volume. The mean value of the peroxidase activity of the serum was 5.3 U/L in the ring signal wounds, 4.8 U/L in the non-ring wounds, and 5.1 U/L in the rats without wounds (Figure 3(a)). There was no significant difference among the three groups ($p=0.481$). These results suggested that peroxidase activity in serum did not affect to the peroxidase signal detected by wound blotting.

Sources of peroxidase activity detected by wound blotting

The peroxidase activity was detected at the area with necrotic cells and inflammatory cells in the wound tissue (Figure 4(a)). MPO was localized in the inflammatory cells and the

top layer of the wound tissue with necrotic cells. GPx3 was detected in the blood vessels and collagen fibers, while no peroxidase activity was detected in either of the latter two areas.

Peroxidase activity signal detected by wound blotting and histopathology

To clarify the histopathology related to the peroxidase activity signals, the histological characteristics were compared between the wounds with ring and non-ring signals. From the histological description, the following features were extracted for the analysis: the presence of extravasation of red blood cells, bleeding, fibrin, cell necrosis, cell debris, karyolysis, denatured cells, inflammatory cells, fibroblast-like cells, collagen fibers, hyalinization, and edema. Because types of inflammatory cells could not be identified in most of the tissue sections due to necrosis, inflammatory cells were not classified.

Inflammatory cells and fibroblast-like cells were detected in all wounds. Among 13 wounds with a ring signal, cell necrosis, cell debris, karyolysis, and denatured cells were detected in 12 wounds, 4 wounds, 5 wounds, and 9 wounds, respectively (Figure 5). No histological feature was detected significantly higher/lower in the wounds with ring signal than the wounds with non-ring signal. Denatured or ruptured cells were observed frequently in the wounds with a ring signal than the wounds with non-ring signal. The wounds showed three types of staining for peroxidase activity: dense, round, and dispersed signals (Figure 4(b)). The dense signals consisted of granular signals with dark color. The round signals were well-circumscribed, and sometimes included dense signals inside of it. We assumed that dense and round signals indicated intracellular peroxidase. The wounds with a ring signal showed the dispersed peroxidase activity at the top layer of the wounds, where many inflammatory cells accumulated. Because the dispersed peroxidase activity was

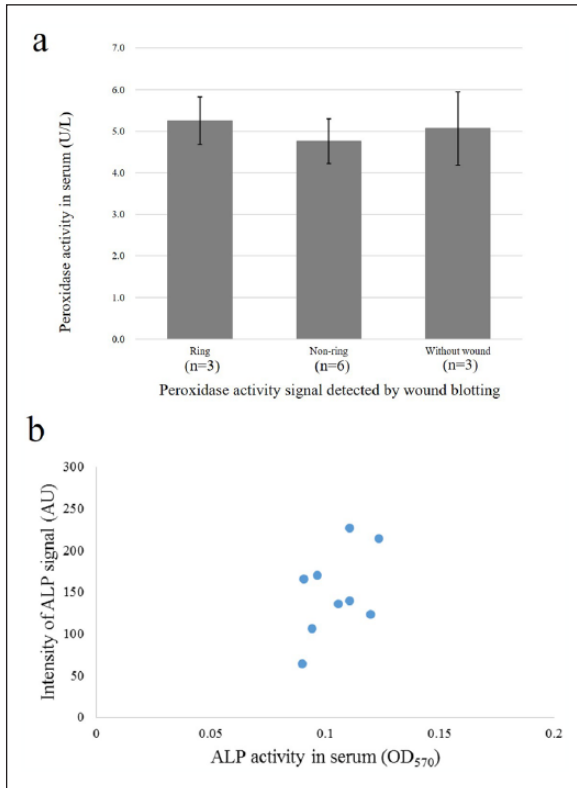


Figure 3. Peroxidase and alkaline phosphatase activity in serum. (a) The ranges of peroxidase activity levels in serum of wounds with ring and non-ring signals were within that of rats without wounds. There was no significant difference among the three groups ($p=0.481$). (b) The signal intensity of alkaline phosphatase tended to depend on the activity levels of alkaline phosphatase in serum. A total of 11 samples were excluded due to hemolysis or an insufficient volume. There was no significant correlation between the ALP activity levels in serum and the intensity of ALP activity detected by wound blotting ($p=0.179$).

observed around the dense or round signals, it was assumed that the dispersed peroxidase indicated extracellular peroxidase, suggesting its deviation or secretion. Thus, the presence of secreted or deviated peroxidase was evaluated based on the enzyme histochemistry. Wounds with a secretion or deviation of peroxidase from cells were observed more frequently in the wounds with a ring signal than in the wounds with a non-ring signal ($p < 0.001$; Table 2).

ALP activity

The mean intensity of the ALP activity signal increased from PWD 1 to PWD 7 and decreased from PWD 7 to PWD 10 in both young and aged rats (Figure 1(d)). Signal intensity of 150 was the threshold used to divide the signals into two groups for the analysis. The ALP signal tended to show a higher intensity in the latter phases of wound healing.

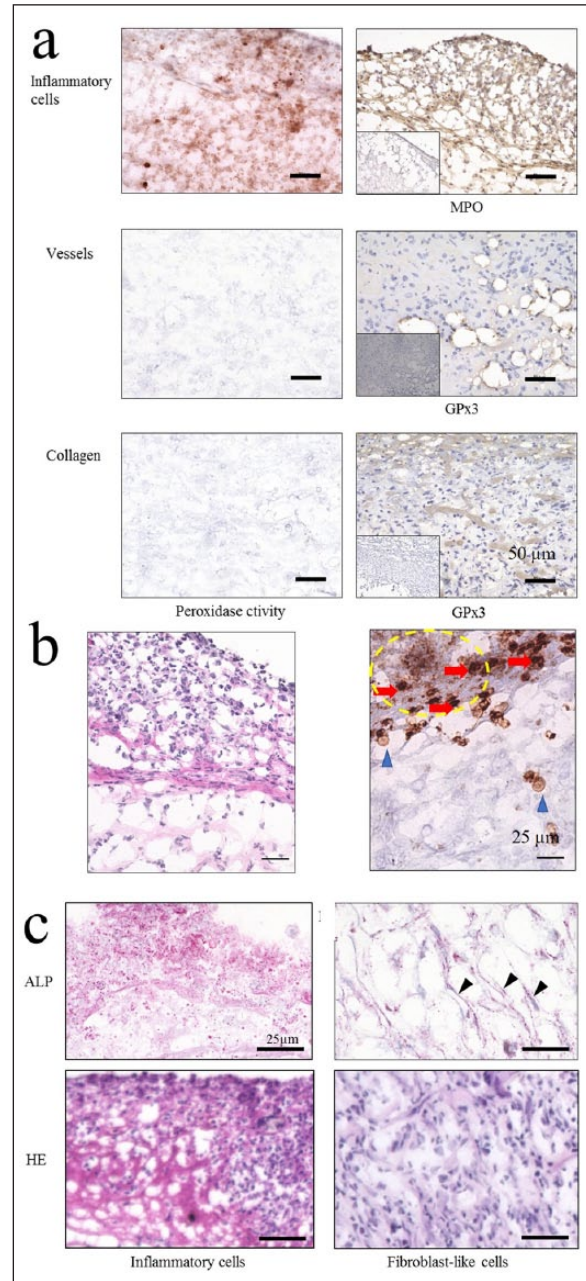


Figure 4. Sources of peroxidase and alkaline phosphatase activity. The left panels show peroxidase activity, and the right panels show myeloperoxidase (MPO) and glutathione peroxidase (GPx) 3. (a) The small windows in the left bottom are the negative control. (b) The left panel shows hematoxylin and eosin (HE) staining, and the right panel shows peroxidase activity in the sections that were collected from the same wound with a ring signal. The yellow dot circle indicates dispersed peroxidase activity (right). The red arrows indicate dense signals, and the blue arrow heads indicate round signals. (c) The upper panels show alkaline phosphatase (ALP) activity, and the lower panels showed HE. The left panels show the inflammatory cells, and the cells in the right panels are fibroblast-like cells. Arrowheads indicate ALP activity in fibroblast-like cells.

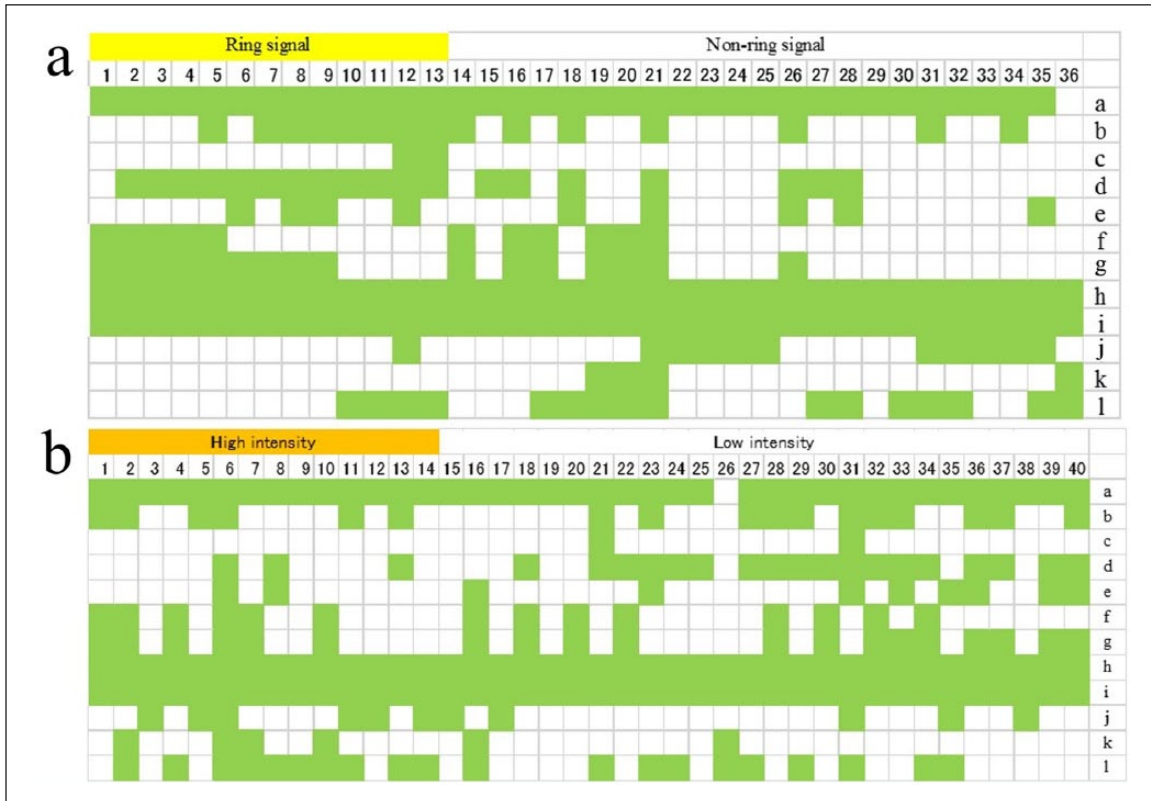


Figure 5. Peroxidase and alkaline phosphatase activity signals and histology. Each column shows an individual wound. From the histological description made by a medical technologist, several features indicated below were extracted for the analysis. In the line (a)–(l), a green color shows that the wound had cells or histology indicating the following: (a) extravasation of red blood cells, (b) bleeding, (c) fibrin, (d) necrosis, (e) cell debris, (f) karyolysis, (g) denatured cells, (h) inflammatory cells, (i) fibroblast-like cells, (j) collagen, (k) hyalinization, and (l) edema. (a) The peroxidase activity signals were classified into ring and non-ring signals. (b) The mean intensity of alkaline phosphatase (ALP) activity signals was measured using ImageJ software and the wounds were ordered according to the intensity. Inflammatory cells and fibroblast-like cells were detected in all wounds. Among 13 wounds with a ring signal, cell necrosis, cell debris, karyolysis, and denatured cells were detected in 12 wounds, 4 wounds, 5 wounds, and 9 wounds, respectively.

Table 2. Relationship between the ring signal of the peroxidase activity and the deviation/secretion of peroxidase in the wound tissue.

		Deviation or secretion		
		Yes	No	
Peroxidase detected by wound blotting	Ring	11	2	$P < 0.001$
	Non-ring	4	17	

The signals of peroxidase activity were classified into a ring signals and a non-ring signals. A total of 40 wounds were sampled, and four samples were excluded because of bleeding of the wounds. The frozen tissue sections were stained for the peroxidase activity, and the dispersed peroxidase activity around inflammatory cells and cell debris were evaluated as the deviation or secretion of peroxidase. Fisher’s exact test.

However, the difference in the intensity of the ALP signal between individual rats was not small.

The serum samples with problems associated with either hemolysis or an insufficient volume were excluded from the measurements. The wounds with the high ALP activity in

serum tended to show the highest ALP signals (Figure 3(b)). However, there was no significant correlation ($p = 0.179$).

In the wound tissue, ALP activity was detected at the area with cell debris, in inflammatory cells, and in fibroblast-like cells (Figure 4(c)). Inflammatory cells and fibroblast-like cells were identified in all wounds. Collagen fibers were detected in 6 of 14 wounds with a high-intensity ALP signal (Figure 5). There were no particular histological features that were observed more frequently in the wounds with a high-intensity ALP signal.

Any results other than the ring and non-ring signals of peroxidase did not show statistically significant difference.

Discussion

This is the first study to demonstrate that a wound blotting analysis of the peroxidase activity can estimate strong inflammation within tissues.

The dot blot experiments with peroxidase suggested that the peroxidase activity level could not be evaluated by the signal intensity. This study therefore used signal distributions as

an indicator of the peroxidase activity level. The peroxidase activity level of a ring signal was assumed to be higher than that of a non-ring signal. We confirmed that the ring and non-ring signals of peroxidase activity did not depend on the perimeter of a wound in our preliminary experiments (data not shown). The ring signals were detected in the early phases of wound healing, in which the wound tissue showed abundant infiltration of inflammatory cells. The sources of peroxidase activity were assumed to be MPO and GPx3 based on the fact that wound blotting can detect only extracellular enzymes. In addition, GPx3 in serum was assumed to affect the peroxidase activity level in wound exudate because the peroxidase activity in serum, which includes GPx3, was not related to the phases of wound healing. Therefore, MPO is the most likely source of peroxidase activity detected by wound blotting. The histological analysis suggested that the ring signal of the peroxidase activity could originate from either deviated or secreted MPO, thus indicating a strong inflammatory reaction. Our results consisted with Schiffer's study which reported that MPO can be a biomarker of infected wounds using wound exudates.²⁴ However, our results indicated that peroxidase activity detection by wound blotting method could be used to assess not only for infected wounds but also wounds with high inflammatory reaction such as critical colonization.

The sources of non-ring signals were not identified in this study. The tissue sections of wounds with a non-ring signal showed positive peroxidase activity in inflammatory cells, while MPO was scarcely detected. Furthermore, GPx3 was positive in collagen fibers and blood vessels, while the peroxidase activity was negative. There is the possibility that the peroxidase activity was too weak to be detected at sites other than those with inflammatory cells. A previous study reported that peroxidase would contribute to collagen biosynthesis²⁵ and angiogenesis,²⁶ thus supporting the possibility of peroxidase activity in collagen fibers and blood vessels.

The results of the dot blot experiments for ALP activity suggested that the ALP activity level could be evaluated by the signal intensity. The result that the mean intensity of ALP activity was highest on PWD 7 is in line with that of a previous study that reported an increase in the extracellular ALP activity in the regions of granulation tissue formation.²² The histological analysis of ALP activity indicated that the deviation of ALP from inflammatory cells and secretion from fibroblast-like cells might cause a strong ALP signal in the exudate. However, there was no statistically significant difference. Further study is needed to standardize the ALP intensity in individuals because the ALP signal detected by wound blotting depended on the ALP activity in serum.

We used both young and aged rats in this study. Their wound appearance (i.e. slough disappearance, wound contraction, and wound closure) showed different time course; however, they had common histological features when their peroxidase signals were the ring.

This study has several limitations. First, the wound model used in this study was an excisional skin wound. The full-thickness wound model used in this study replicated healing

by secondary intention. An excisional wound is generally used in studies that focus on the healing of cutaneous open wounds. In contrast, PUs are ischemia/reperfusion injuries. In PUs, the surrounding and underlying tissue may also have been damaged. This difference could affect the infiltration of inflammatory cells that contribute to the wound healing. Second, the study used only full-thickness wounds. Because the layer of dermis remains in a partial-thickness wound, the wound undergoes a process of regeneration, not remodeling. In addition, partial-thickness wounds have a large number of blood vessels, fibroblasts, and collagen fibers compared to full-thickness wounds at the beginning of the wound-healing process. Therefore, the peroxidase activity may originate from not only MPO but also other kinds of peroxidase. However, inflammatory cells play similar roles in both types of wounds. Further study is therefore needed to clarify the pathophysiology of wounds related with the peroxidase activity in partial-thickness wounds.

We conclude that the ring signals of peroxidase activity detected by wound blotting could be a candidate marker to evaluate wound histopathology as a non-invasive and point-of-care assessment.

Animal welfare

This study followed the national and institutional guidelines for humane animal treatment and complied with relevant legislation.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval

Ethical approval for this study was obtained from the Animal Experimentation Committee of the School of Medicine, the University of Tokyo (医-P14-087).

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